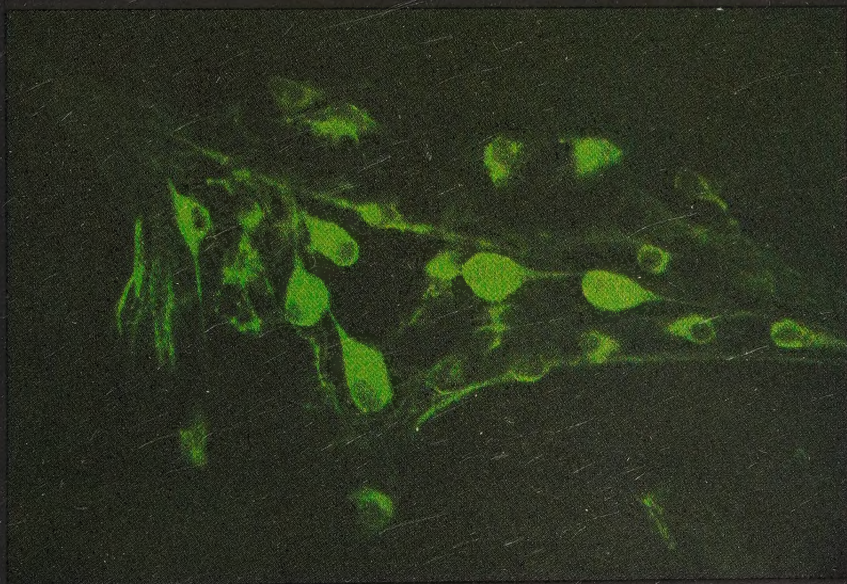


PROJECTIONS OF ENTERIC PEPTIDE-CONTAINING NEURONS IN THE RAT



EVA EKBLAD

Diss. Lund

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**PROJECTIONS OF ENTERIC
PEPTIDE - CONTAINING NEURONS
IN THE RAT**

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Title and subtitle

Projections of enteric peptide-containing neurons in the rat

Abstract The distribution, origin and projections of enteric nerve fibers containing vasoactive intestinal peptide (VIP), neuropeptide Y (NPY), galanin, substance P (SP), calcitonin gene-related peptide (CGRP), gastrin releasing peptide (GRP), somatostatin and enkephalin were studied in the rat small and large intestine. The origin of these nerve fibers was examined by immunocytochemistry after extrinsic denervation. This revealed that the bulk of the peptide-containing nerve fibers are intramural in origin, each population showing its own characteristic topographic distribution. The projections of each neuronal population were studied using a combination of immunocytochemistry and immunocytochemistry after local severing of nervous pathways (myectomy, transection, intestinal clamping) by examining the subsequent axonal degeneration. This revealed that myenteric neurons issue predominantly descending projections to other myenteric ganglia and to the muscle layers. Enkephalin-containing neurons in both small and large intestine and SP neurons in the large intestine issue ascending projections. Submucosal neurons project to other submucosal ganglia and to the mucosa and submucosa. Most of them issue both ascending and descending projections except for those storing CGRP, VIP/NPY (small intestine) or VIP (large intestine) which issue ascending projections only. The projection distances varied considerably between the different neuronal populations, the majority were in the range of 4-10 mm. Myenteric GRP and galanin neurons in the small intestine issued the longest ones, 20 and 15 mm, respectively. The shortest were those from myenteric VIP/NPY neurons and submucosal galanin and GRP neurons in the small intestine and submucosal VIP neurons in the large intestine which were 2 mm in length. The plasticity of enteric GRP neurons was studied in rat small intestine at different time intervals up to 60 weeks after myectomy. Fine varicose GRP fibers gradually reinnervated the portion anally to the lesion beginning at 20 weeks. During this period also the circular muscle of the myectomized segment became reinnervated but these GRP fibers were coarse and more numerous compared to normal (hyperinnervation).

Key words Neuropeptides, enteric neurons, neuronal projections, VIP, SP, NPY, CGRP, GRP, somatostatin, enkephalin, neuronal plasticity, reinnervation.

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Date 1986 - 11 - 15

Projections of Enteric Peptide-containing
neurons in the rat

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Lund, Sweden

Lund 1986

Front page: Myenteric ganglia in rat colon stained with neurofilament antiserum. Whole mount. X175

To Anders and my parents

The present thesis is based on the following papers, which will be referred to by their Roman numerals.

- I GRP neurons in the rat small intestine issue long anal projections.
E. Ekblad, R. Ekman, R. Håkanson and F. Sundler.
Regulatory Peptides 9:279-287, 1984.
- II Galanin nerve fibers in the rat gut:
Distribution, origin and projections.
E. Ekblad, Å. Rökaeus, R. Håkanson and F. Sundler.
Neuroscience 16:355-363, 1985.
- III Projections of peptide-containing neurons in rat small intestine.
E. Ekblad, C. Winther, R. Ekman, R. Håkanson and F. Sundler.
Neuroscience. In press.
- IV Projections of peptide-containing neurons in rat colon.
E. Ekblad, R. Ekman, R. Håkanson and F. Sundler.
In manuscript.
- V Reinnervation in the gut: Return of GRP nerve fibers in rat small intestine after local removal of myenteric ganglia.
E. Ekblad, R. Ekman, R. Håkanson and F. Sundler.
Neuroscience. Submitted.

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INTRODUCTION

The autonomic nervous control of visceral functions is exerted by "central" (parasympathetic, sympathetic and sensory) as well as by local neurons (Fig. 1). The digestive tract is conspicuously rich in local neurons. The neurons that are intrinsic to the gut wall are traditionally referred to as the enteric nervous system. Processes such as absorption, secretion and coordinated peristaltic activity of the smooth muscle resulting in propulsion of the content are to a great extent regulated by intramural nerve reflexes (for reviews see Kosterlitz and Watt 1975, Costa and Furness 1982, Cooke 1986).

Extrinsic nervous control

Of the neuronal elements in the gut extrinsic fibers constitute a minority population. They reach the gut via the vagus, the splanchnic nerves and the sacral nerves. The fibers, which terminate predominantly in the intramural ganglia and around blood vessels, have their cell bodies in the jugular and nodose ganglia, the abdominal sympathetic ganglia, thoracolumbar and sacral spinal ganglia and in the brain stem (dorsal vagal motor nucleus) and sacral spinal cord (for reviews see Schofield 1968, van Driel and Drukker 1973, Gabella 1979, Gershon 1981). The vagus participates in the control of both motor and secretory functions in the gut and the vagal control of motor function plays a greater role in the proximal part of the gut (esophagus and stomach) than in the more distal part. Cervical vagotomy impairs but does not abolish esophageal motility (Mukhopadhyay and Weisbrodt 1975). Similarly, gastric emptying is impaired but not abolished by vagotomy (Kalbasi et al 1975). Vagotomy impairs also mucosal functions as exemplified by the pronounced reduction in gastric acid output in the rat (c.f. Håkanson et al 1982). The effects of vagotomy was suggested by Oki and Daniel (1979) to be associated with disintegration of a proportion of the synaptic endings in the intramural ganglia.

The sympathetic nerves mediate the extrinsic inhibition of gut motility. The effects are thought to be exerted at least partly via inhibition of intrinsic cholinergic motor neurons (Furness and Costa 1974). Sensory afferents reach the gut via the vagal and splanchnic nerves. Dendritic nerve endings are thought to act as mechano-, thermo- and chemoreceptors, activating intramural reflexes (c.f. Cooke et al 1986). However, much information is still missing on the identity and precise location of receptor sites and pathways integrating the control of motility and secretion.

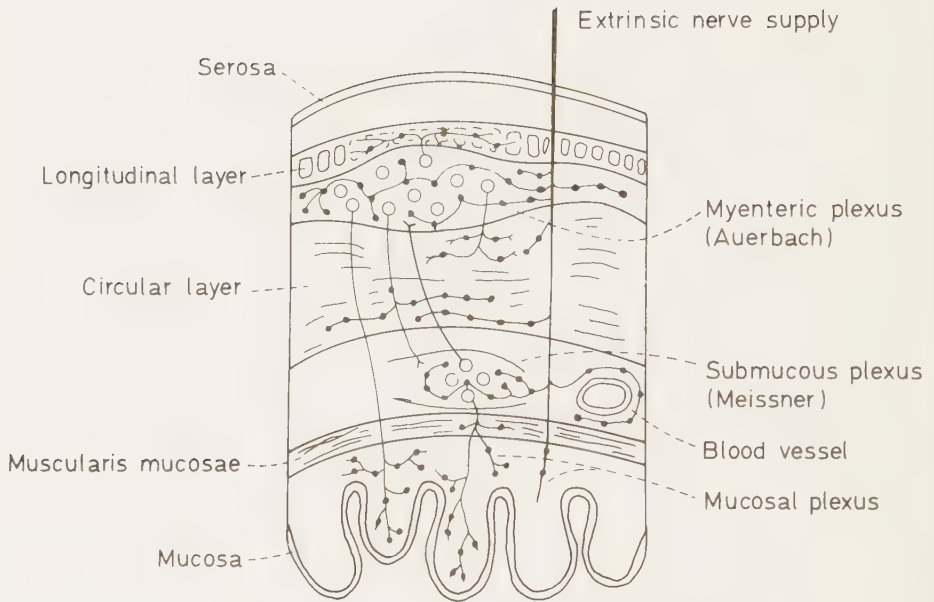


Fig. 1. Schematic illustration of the organization of extrinsic nerve fibers (parasympathetic, sympathetic and sensory) and intramural ganglia in the gut. The intramural ganglia consist of myenteric (Auerbach) plexus and submucous (Meissner) plexus.

Intrinsic nervous control

The intrinsic fibers which represent most of the nervous elements in the gut have their cell bodies in the intramural ganglia.

The intrinsic neurons can be subdivided on the basis of:

1) Location. The myenteric ganglia contain large collections of nerve cell bodies and are situated between the outer longitudinal and inner circular muscle layers. The ganglia and the interconnecting nerve strands form a continuous meshwork with nerve cell bodies accumulated at the nodes of the meshwork. The size of the ganglia and the shape of the meshwork vary between the different parts of the gut and between different species (Schofield 1968, Gabella 1979). The number of myenteric nerve cell bodies in the small and large intestine ranges from 10000 to 20000 /cm² as estimated in the rat, guinea pig and cat (for review see Gabella 1979). The projections from myenteric neurons terminate mainly in the smooth muscle and within other myenteric ganglia. In addition, some mainly myenteric neurons project to extrinsic ganglia (Costa and Furness 1983, Schultzberg and Dalsgaard 1983). The submucous ganglia and their interconnecting nerve bundles form a meshwork similar to that of the myenteric ganglia. However, the meshes are larger and less regular and the ganglia are smaller (Schofield 1968, Gabella 1979). In the guinea pig ileum and colon submucous neurons are about 4000 /cm² (Ohkubo 1936). The projections of submucous neurons terminate preferentially in the mucosa and submucosa. In total the number of neurons in the intramural ganglia of the cat small intestine is approximately 6 millions (Sauer and Rumble 1946).

2) Possible functions. The fundamentals for an intact integrative circuitry, i.e. sensory neurons, interneurons and secreto-motor neurons, are present in the enteric ganglia. Several nerve reflexes are involved in the propulsion of a bolus, one is referred to as the enteric ascending excitatory reflex and another one as the enteric descending inhibitory reflex (for reviews see Kosterlitz and Watt 1975, Costa and Furness 1982). Reflexes maintaining mucosal functions are suggested to start by mechanical or chemical activation of mucosal sensory receptors, which excite interneurons and secreto-motor neurons in the submucous ganglia (c.f. Cooke 1986).

3) Electrophysiological properties. Four different types of enteric neurons have been recognized so far: type 1 or S cells, type 2 or AH cells, type 3 and type 4 cells (Nishi and North 1973, Hirst et al 1974, c.f. Wood 1983). The

classification is based upon the intracellularly recorded electrical behaviour of the nerve cell bodies. Clearly the data available at present underestimate the true number of functionally different neuronal populations.

4) Morphology. A number of conventional histological staining techniques, such as methylene blue and various silver staining procedures, have been used to study the morphology of the enteric nervous system. Three types of neurons in the intramural ganglia were described by Dogiel in his classical studies (Dogiel 1895, 1896, 1899). Type I cells are characterized by numerous short dendrites and a single long axon. Type II cells have a short axon and fewer and longer dendrites. Type III cells have a long axon and dendrites of intermediate length. Further attempts to classify enteric neurons have been performed by Stach (1981), who extended Dogiel's classification with a 4th neuron population. This myenteric neuron is characterized by numerous dendrites and an axon running vertically between the myenteric ganglia and submucous ganglia. There is little doubt that also this classification underestimates the true number of different neuronal populations.

5) Ultrastructure. The neurons in the gut have been ultrastructurally classified according to the size, morphology and electron density of the vesicles present in the terminals. The different vesicular populations have been described as small clear vesicles, small granular vesicles, medium-sized granular vesicles, large granular vesicles, large opaque vesicles, flattened vesicles or ring vesicles (Baumgarten et al. 1970, Gabella 1972, Cook & Burnstock 1976, Llewellyn-Smith 1983). Although these various types of vesicles usually occur in different nerve endings, two or more may coexist in the same terminal. It is now generally held that dense-cored vesicles (granular vesicles) contain neuropeptides (Baumgarten et al 1970, Polak and Bloom 1978, Sundler et al 1982). If also clear vesicles contain peptides is still an open question.

6) Histochemical properties. Specific histochemical techniques have been developed to identify the individual neuronal populations on the basis of their transmitter content. These techniques include staining for cholinesterase activity, monoamine fluorescence histochemistry and immunocytochemistry. Based on the results of such techniques enteric neurons have been claimed to store acetylcholine (Coupland and Holmes 1957, Furness et al 1983), serotonin (Costa et al 1982), GABA (Baetge and Gershon 1986, Jessen et al 1986) and a great number of neuropeptides (Polak and Bloom 1978, Sundler et al 1978, Håkanson and Sundler 1979, Schultzberg et al 1980, Sundler et al 1980, Furness and Costa 1982). In fact the digestive tract seems to be richer in neuropeptides than any

other tissue outside the brain. For several of the peptides a tentative functional role in gut regulation has been proposed (for a review see Makhoulf 1985). So far, convincing evidence for such a role has been obtained only for the tachykinins (Leander et al 1981, Barthó and Holzer 1985, Gintzler et al 1985).

Neuropeptides in the gut (Table 1)

Calcitonin gene-related peptide (CGRP). CGRP is 37 amino acids long and arises by alternative processing of the calcitonin gene mRNA transcript (Amara et al 1982, Rosenfeld et al 1983). CGRP occurs together with SP in primary sensory neurons in a number of peripheral organs (c.f. Sundler et al 1985a). In the gut CGRP exists in intrinsic neurons mainly distributed in the myenteric ganglia Clague et al 1985) and together with SP in perivascular nerve fibers, probably of sensory origin (Sundler et al 1985a). CGRP is a potent vasodilator (Brain et al 1985). Few reports have dealt with the actions of CGRP in the gut. It has been reported to cause dose-dependent contractions of the guinea-pig ileum (Ghatei et al 1984, Tippins et al 1984).

Enkephalins. Leu- and met-enkephalin were isolated from brain extracts based on their properties as opiate receptor agonists (Hughes et al 1975). Enkephalin neurons in the gastrointestinal tract occur preferentially in the myenteric ganglia (Elde et al 1976, Alumets et al 1978, Schultzberg et al 1980). There are three known opioid peptide precursors in the body: proopiomelanocortin, proenkephalin A and proenkephalin B (c.f. Numa 1985). Neuronally localized enkephalins in the gut probably arise from either proenkephalin A or B. This assumption is based on the fact that components of both precursors have been localized immunocytochemically to neuronal elements in the gut (Giraud et al 1984, Kobayashi et al 1984, Vincent et al 1984, Costa et al 1985, Wang and Lindberg 1986). Enkephalins have a morphin-like effect on the digestive tract i.e. a presynaptic inhibition of the cholinergic neurons mediating contraction (van Nueten et al 1977, Waterfield et al 1977) as well as a direct contractile effect on the muscle cells (Bitar and Makhoulf 1982, Hellström 1985).

Galanin. This 29 amino acid peptide with N-terminal glycine and C-terminal alanine amide was isolated from the porcine upper small intestine (Tatemoto et al 1983). Galanin nerve fibers have been demonstrated both in the central and peripheral nervous systems (Rökaeus et al 1984). Galanin has been shown to elicit different effects on different smooth muscle preparations, including a neuromodulatory (inhibitory) effect on the guinea pig taenia coli and a direct contractile effect on rat jejunal longitudinal muscle (Ekblad et al 1985).

Table 1. Amino acid sequences of neuropeptides in the gut.

Calcitonin gene-related peptide:

Ala-Cys-Asp-Thr-Ala-Thr-Cys-Val-Thr-His-Arg-Leu-Ala-Gly-Leu-Leu-Ser-Arg-Ser-Gly-Gly-Val-Val-Lys-Asn-Asn-Phe-Val-Pro-Thr-Asn-Val-Gly-Ser-Lys-Ala-Phe-NH₂

Enkephalins:

Met-enk: Tyr-Gly-Gly-Phe-Met
 Leu-enk: Tyr-Gly-Gly-Phe-Leu

Galanin:

Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-His-Ala-Ile-Asp-Asn-His-Arg-Ser-Phe-His-Asp-Lys-Tyr-Gly-Leu-Ala-NH₂

Gastrin releasing peptide:

Ala-Pro-Val-Ser-Val-Gly-Gly-Thr-Val-Leu-Ala-Lys-Met-Tyr-Pro-Arg-Gly-Asn-His -Trp-Ala-Val-Gly-His-Leu-Met-NH₂

Neuropeptide Y:

Tyr-Pro-Ser-Lys-Pro-Asp-Asn-Pro-Gly-Glu-Asp-Ala-Pro-Ala-Glu-Asp-Leu-Ala-Arg-Tyr-Ser-Ala-Leu-Arg-His-Tyr-Ile-Asn-Leu-Ile-Thr-Arg-Gln-Arg-Tyr-NH₂

Somatostatin:

Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys

Substance P:

Arg-Pro-Lys-Pro-Gln-Phe-Phe-Gly-Leu-Met-NH₂

Vasoactive intestinal peptide:

His-Ser-Asp-Ala-Val-Phe-Thr-Asp-Asn-Tyr-Thr-Arg-Leu-Arg-Lys-Gln-Met-Ala-Val-Lys -Lys-Tyr-Leu-Asn-Ser-Ile-Leu-Asn-NH₂

Gastrin releasing peptide (GRP). GRP, a 27 amino acid peptide first isolated from the porcine non-antral gastric mucosa (MacDonald et al 1979), is thought to be the mammalian counterpart of bombesin, a 14 amino acid peptide originally isolated from amphibian skin (Anastasi et al 1971). GRP-immunoreactive nerve fibers are numerous in the gut (Moghimzadeh et al 1983), but have been demonstrated also in other peripheral organs such as the genito-urinary tract (Stjernquist et al 1986) and the respiratory tract (Uddman et al 1984). Bombesin/GRP produces a wide variety of effects such as stimulation of neurohormonal peptide release, gastric acid secretion, pancreatic enzyme secretion and gall bladder and intestinal smooth muscle contraction (for reviews on the action of bombesin see Bertaccini et al 1974, Erspamer and Melchiorri 1975).

Somatostatin (Som). Som, a 14 amino acid peptide, was originally isolated from the hypothalamus (Brazeau et al 1973) by virtue of its ability to inhibit the release of growth hormone. In addition, Som inhibits many other release processes, both exocrine and endocrine (c.f. Raptis et al 1978). In the gut Som has a dual localization, in endocrine cells and in nerve fibers (Hökfelt et al 1975, Alumets et al 1977). The actions of Som on intestinal motility are thought to be neuromodulatory. Thus, Som reduces the amplitude of ileal contractions elicited by cholinergic nerve stimulation without antagonizing the action of exogenously applied acetylcholine (Guillemin 1976). Som seems to act also on non-adrenergic inhibitory neurons (Furness and Costa 1979) possibly thereby interfering with peristalsis without having a direct effect on the smooth muscle cells.

Substance P (SP). SP is an 11 amino acid peptide belonging to the tachykinin family of peptides (Pernow 1983). Although it was discovered many years ago in extracts of intestine (von Euler and Gaddum 1931) it was not isolated and sequenced until 1971 (Chang et al 1971). SP (and possibly also other tachykinins) is widely distributed in the body and is known to occur in a population of primary sensory neurons (c.f. Pernow 1983, Sundler et al 1985a). Based on the cDNA sequence two different bovine brain SP precursors (α - and β -preprotachykinin) have been predicted (Nawa et al 1983). The two SP mRNAs seem to arise by alternative processing of a single primary RNA transcript. The α -preprotachykinin gives rise to a second tachykinin, neurokinin A (NKA), in addition to SP. There is good evidence that β -preprotachykinin is expressed in gut neurons (c.f. Sundler 1985a). Whether α -preprotachykinin is expressed in gut neurons remains to be clarified.

In the gut SP has a dual localization in neuronal elements and in endocrine cells (Nilsson et al 1975, Sundler et al 1977). The intramural SP neurons in the gut occur predominantly in myenteric ganglia. There is strong evidence that SP is a transmitter in primary afferent neurons (Lembeck 1953, Otsuka et al 1975, Otsuka and Konishi 1983). SP dilates blood vessels and contracts non-vascular smooth muscle (c.f. Pernow 1983).

Vasoactive intestinal polypeptide (VIP). VIP, a 28 amino acid peptide, was originally isolated from porcine duodenum (Said and Mutt 1970). It is a member of the glucagon peptide family. VIP is abundantly distributed in neuronal elements of the brain and peripheral organs (c.f. Polak and Bloom 1982). The digestive tract, especially the sphincteric regions, is richly supplied with VIP nerve fibers of intrinsic origin (Alumets et al 1979, Malmfors et al 1981). VIP neurons contain also the structurally related peptide PHI (Yanaihara et al 1983, Ekblad et al 1984). This coexistence can be anticipated since the two peptides derive from the same precursor (Itoh et al 1983) (see below). The major effects of VIP are vasodilatation (c.f. Said 1982), relaxation of non-vascular smooth muscle (Said 1973, Kachelhoffer et al 1976) and stimulation of exocrine gland secretion (Christophe and Robberecht 1982). The effects of PHI are reminiscent of those of VIP (Brennan et al 1982, Ghiglionone et al 1982, Anagnostides et al 1984).

Synthesis and coexistence of neuropeptides

Neuropeptides are synthesized in the rough endoplasmic reticulum as large precursor molecules which are progressively cleaved in smaller and smaller fragments. Much of the intracellular processing takes place in the secretory granules which also serve to store the processed material (Håkanson and Sundler 1983, Brownstein 1985).

The main processing of the neuropeptides is in the form of proteolytic cleavages, preferentially at positions of paired basic amino acid residues giving rise to a number of potentially bioactive split products. As a result several bioactive peptides will coexist in the same neuron. Such coexistence of peptides generated from the same precursor may be predicted (for a review see Sundler et al 1985b). An example of predictable coexistence is VIP and PHI which share a common precursor (Itoh et al 1983). Also the coexistence of SP and NKA is explained by the fact that they have a common precursor (Nawa 1983). Somewhat unexpectedly, however, also peptides originating from different precursor molecules have been found to coexist in the same neuron. Examples of this type of unexpected coexistence of peptides in the gut neuronal elements are VIP/NPY (Ekblad et al 1984), VIP/galanin (Melandar et al 1985), SP/CGRP

(Gibbins et al 1985, Sundler et al 1985a) and NPY/Som/CCK/CGRP (Furness et al 1985). Peptides may also occur together with classical transmitters such as noradrenaline, acetylcholine (ACh) or serotonin. The coexistence of NPY/noradrenaline (Ekblad et al 1984) in extrinsic (sympathetic) fibers and SP/serotonin (Legay et al 1984), SP/ACh and CCK/Som/NT/NTS (Furness et al 1984) in intrinsic neurons may serve as examples.

AIM OF THE STUDY

The present study focuses on two problems:

- 1) The origin and projection patterns of enteric peptide-containing neurons.
- 2) The plasticity of enteric neurons as exemplified by GRP neurons in the rat small intestine.

METHODS

Animals. Adult female Sprague-Dawley rats (150-200 g) were used.

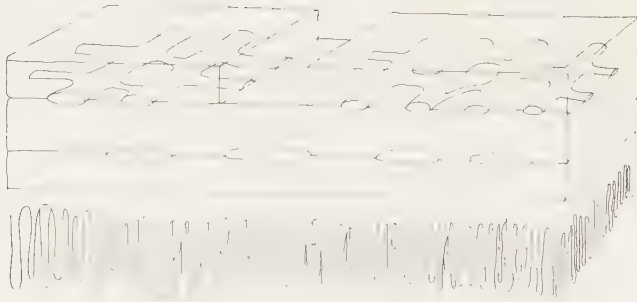
Denervation procedures

Elimination or severing of extrinsic nerves: 1) Bilateral subdiaphragmatic truncal vagotomy and pyloroplasty. 2) Chemical sympathectomy by 6-hydroxydopamine. 3) Nerve crush by clamping of mesenteric blood vessels and surrounding mesentery of an intestinal loop approximately 10 cm in length.

Severing of intrinsic nervous pathways: 1) Circumferential myectomy i.e. removal of the outer longitudinal smooth muscle layer with adherent myenteric ganglia from a 10 mm segment of the gut. 2) Transection with end-to-end anastomosis. 3) Nerve crush by circumferential clamping with a pair of forceps (Fig. 2). The animals were killed 8-10 days later if not otherwise stated. The projection patterns of the various peptide-containing neurons were deduced by examination of the subsequent axonal degeneration (Fig. 3). The nerve fiber length was established by measuring the distance from the lesion to the region showing a normal supply of terminals. This distance includes one region lacking immunoreactive terminals and one region showing a gradual increase in the number of nerve terminals. Distances were measured using a micrometer scale inserted in the visual field. If the fiber frequency of the nerve fiber population under investigation is moderate to numerous the shortest measurable projection distance is in the range 0.3-0.4 mm.

In order to study the return of GRP nerve fibers after local removal of myenteric ganglia the animals were killed at various times up to 60 weeks after the operation.

Myectomy



Transection



Clamping

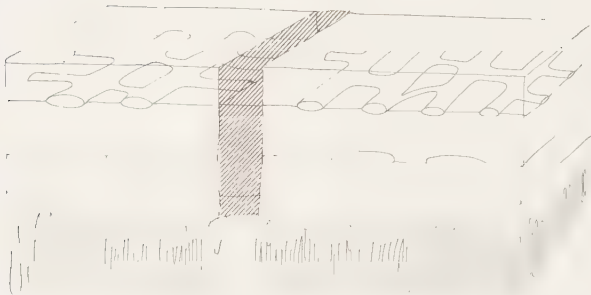


Fig. 2. Procedures used to interrupt enteric nervous pathways. Myectomy involves circumferential removal of 10 mm of the outer longitudinal muscle layer with adherent myenteric ganglia, thus severing the nervous pathways emanating from these ganglia. Transection is followed by the joining with subsequent suturing of the severed ends. Clamping interrupts enteric nervous pathways by crushing the nerves for 3 to 4 mm with a pair of forceps. The two latter procedures severed nervous pathways in both myenteric and submucous ganglia.

Intact enteric nervous pathways



Disruption of nervous pathways

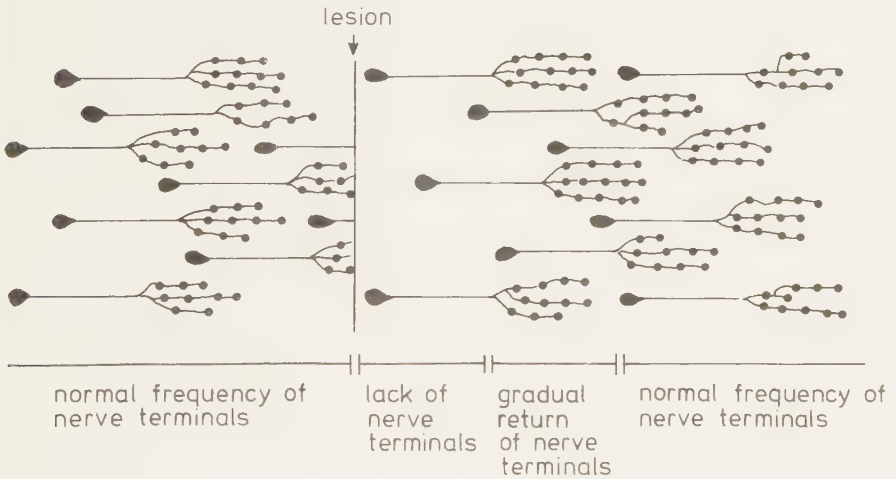


Fig. 3. Diagram illustrating the uni-directional projections of a hypothetical population of enteric neurons and the proposed consequences of local disruption of the pathways. In the diagram it is assumed (perhaps erroneously) that all projections of this hypothetical neuronal population are of the same length. Cell bodies and preterminals have a low neuropeptide content, the nerve terminals are rich in the neuropeptide.

Immunocytochemistry

Tissue specimens from the mid-jejunum and the transverse colon of operated and unoperated rats were fixed in buffered 4% formaldehyde or Stefanini's fixative, rinsed in Tyrode solution containing 10% sucrose and processed for immunocytochemistry either as whole mounts or as cryostat sections. The indirect immunofluorescence method (Coons et al 1955) was used for the immunocytochemical demonstration of the various neuropeptides (Table 2). For controls, antisera that had been inactivated by the addition of excess amounts of antigen (10-100 ug of synthetic peptide/ml diluted antiserum) were used.

Immunocytochemistry is a useful technique for the demonstration of peptides in enteric neurons. However, since antibodies usually recognize only a short sequence within the peptide immunogen the antibodies will crossreact with other peptides having the same or a similar antigenic determinant. Thus, it is important to perform the proper tests to identify crossreaction of the antibodies with other structurally related peptides at the immunocytochemical level (c.f. Larsson 1981). It is also desirable to combine immunocytochemistry with chemical analysis. In principle, to ascertain the identity of a peptide demonstrated by immunocytochemistry it has to be extracted, isolated and sequenced. In practice, however, the identity of a peptide is usually established by the use of HPLC (high pressure liquid chromatography) in combination with immunochemical analysis.

Co-existence of neuropeptides was studied by sequential (VIP/NPY) or double (CGRP/SP and CGRP/VIP) immunostaining (Fig. 4). The fact that nerve fibers often run closely together (companion fibers; see also paper III.) represents a problem in studies of coexistence; the demonstration of coexistence of peptides in nerve cell bodies therefore provides more convincing evidence.

Radioimmunochemistry

The concentrations of GRP, galanin, met-enkephalin, NPY, SP and CGRP were determined by radioimmunoassay. Methodological details are given in the respective papers.

RESULTS AND COMMENTS

General distribution pattern of peptide-containing nerve fibers

Nerve fibers containing VIP, NPY, GRP, galanin, SP, CGRP, Som or enkephalin were found throughout the intestines of the rat. The occurrence and topographic distribution of these neurons are illustrated schematically in Figs. 5 and 6. Most of the nerve fibers in the gut wall were intramural in origin, i.e. their cell bodies were located in the submucous and/or myenteric ganglia. (Extrinsic

fibers included perivascular NPY, CGRP and SP/CGRP fibers). NPY was found in the entire population of intramural VIP nerves in the small intestine; in the large intestine only a small proportion of the VIP fiber population contained NPY. In the colon some intrinsic perivascular VIP fibers contained CGRP.

Projections of enteric neurons (Papers I-IV)

Myenteric neurons. The myenteric neurones were found to project to the myenteric ganglia and to the smooth muscle layers. Except for GRP neurons in the large intestine there was no evidence for projections of myenteric neurons to the mucosa and submucosa. Neurons containing VIP/NPY, VIP (large intestine), GRP, galanin or Som (small intestine) issued descending projections. CGRP neurons issued descending projections in the large intestine, while they projected in both oral and anal directions in the small intestine. SP-containing neurons issued descending projections in the small intestine, whereas ascending projections were issued in the large intestine. Enkephalin neurones gave off ascending projections in both small and large intestine. The projection distances for all these neuronal populations were found to be in the range 2-7 mm, except for galanin and GRP neurons in the small intestine which issued longer projections, approximately 15 and 20 mm, respectively. The results are summarized in Fig. 7.

Submucous neurons. Generally the submucous neurons projected to other submucous ganglia and to the submucosa and mucosa. However, neurons storing VIP/NPY, CGRP (small intestine) or VIP (large intestine) seemed to issue projections also to subjacent circular muscle. GRP-containing neurons projected in both oral and anal directions in both small and large intestine. Galanin, SP and somatostatin neurons also projected in both directions in the small intestine. In the large intestine galanin neurons issued no oro-anal projections. SP neurons in the mucosa and submucosa of the large intestine were too rare to allow any conclusion as to the projection pattern. CGRP neurons issued ascending projections in the small intestine but projected in both oral and anal directions in the large intestine. VIP/NPY-containing neurons gave off ascending projections in the small intestine but issued no oro-anal projections in the large intestine. Neurones storing VIP but not NPY (large intestine) issued ascending projections. Projection distances in the mucosa and submucosa ranged from 2 to 8 mm, the longest one being that of CGRP neurons. The results are summarized in Fig. 7.

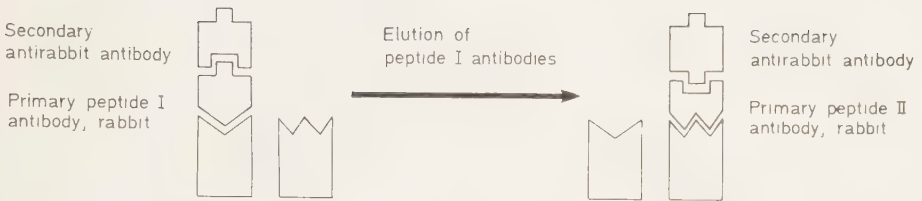
Table 2. Details of the antisera

Antigen	code	raised against	Cryostat sections	Working dilution	Source
VIP	7852	unconjugated pure natural porcine VIP	1:640	1:320	Milab, Malmö Sweden
NPYa)	8404	unconjugated synthetic porcine NPY	1:320	1:160	Milab, Malmö Sweden
	NPYY2	protein-conjugated porcine NPY	1:400	1:200	P.C. Emson, MRC Cambridge
Somatostatin	KB18	protein-conjugated synthetic ovine somatostatin	1:300	1:400	Milab, Malmö Sweden
Met-enkephalin Met-enk b)		protein-conjugated synthetic met-enkephalin	1:320	1:160	R.J. Miller and K.J. Chang Burrroughs Wellcome Research Triangle Park, N.J. USA
Substance P	8107	protein-conjugated synthetic met-enkephalin	1:160	1:80	Milab, Malmö Sweden
	SP8	protein-conjugated synthetic bovine substance P	1:320	1:160	P.C. Emson, Cambridge, U.K.
CGRP	8427	protein-conjugated synthetic CGRP	1:640	1:320	Milab, Malmö Sweden
Galanin	8416	unconjugated synthetic porcine galanin	1:320	1:160	Milab, Malmö Sweden
GRP	R-6902	protein-conjugated synthetic porcine GRP	1:640	1:320	N. Yanaihara, Shizuoka, Japan

a) The two NPY antisera did not cross-react with avian pancreatic polypeptide, bovine pancreatic polypeptide, peptide YY, and produced identical results.

b) The two met-enkephalin antisera recognize also leu-enkephalin.

Sequential staining



Double staining

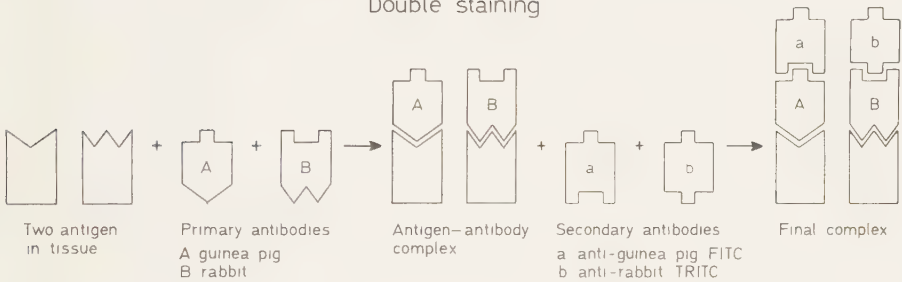


Fig. 4. Immunocytochemical methods used to study the coexistence of two peptides in the same neuron. Sequential staining: Peptide I was visualized by immunofluorescence and photographed. The antibodies were then eluted with potassium permanganate for 30-60 s (Tramu et al 1980). It is essential to verify that both layers of antibodies have been eliminated (to avoid false positive results). This is tested by application of fluoresceinated anti-IgG antibodies. Sections devoid of fluorescence were processed for immunofluorescence of peptide II and again photographed. The details of the elution procedure must be tested individually for each antiserum and for each tissue. The fact that antigens can be destroyed by treatment with potassium permanganate represents a problem. Thus, attempts to demonstrate a second antigen after elution of the antibodies bound to the first antigen may fail for technical reasons (false negative results). Double staining: The section was stained simultaneously with a peptide I antiserum raised in for example guinea pig and a peptide II antiserum raised in for example rabbit. The site of the antigen-antibody reactions was visualized with a FITC-conjugated anti-guinea pig IgG and a TRITC-conjugated anti-rabbit IgG. During examination, filters were shifted to enable selective demonstration of the two fluorophores. Double staining is more convenient and reliable than sequential staining, but requires antibodies raised in different species.

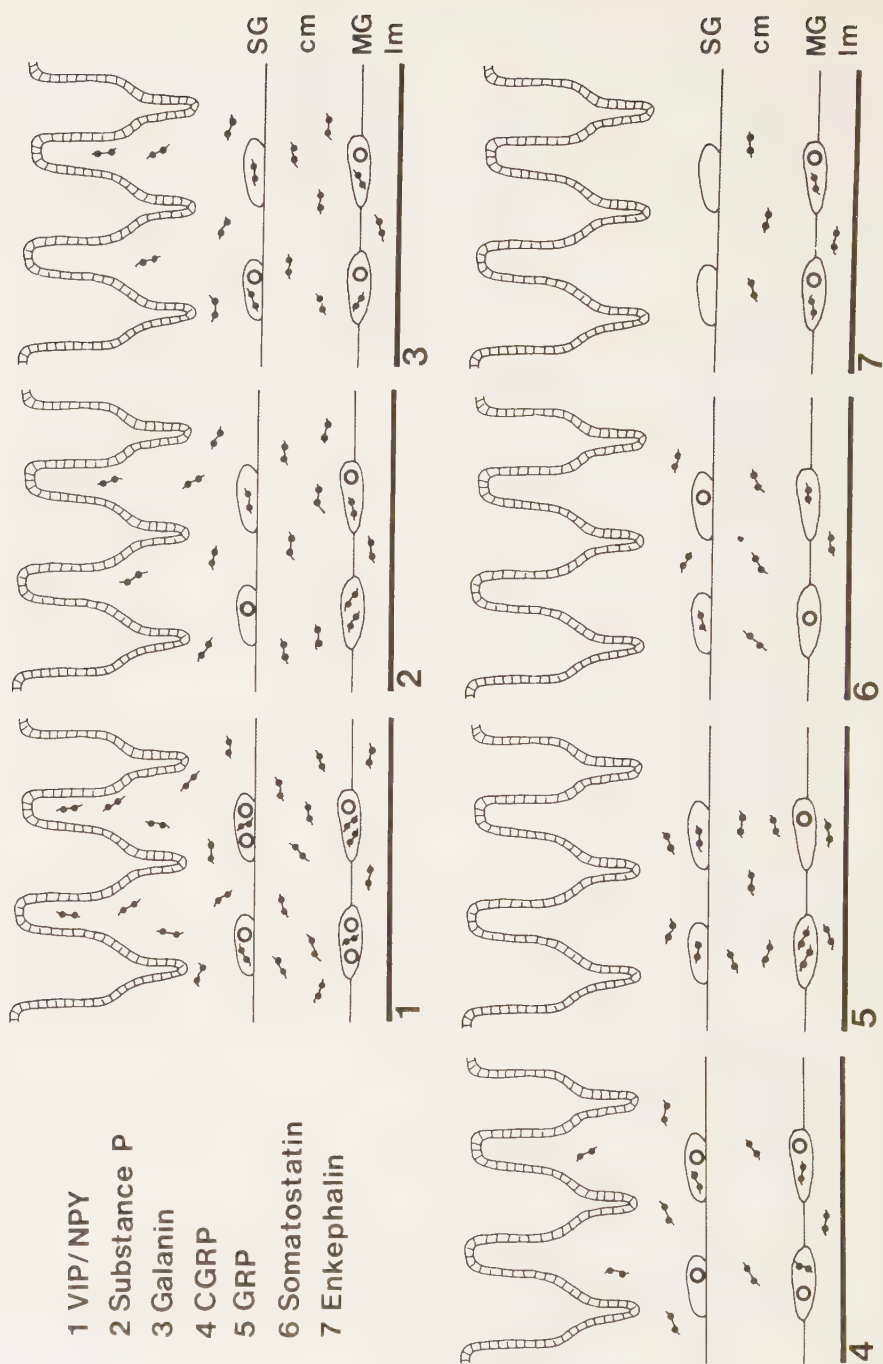


Fig. 5. Schematic outline of the topography and relative density of peptide-containing nerve fibers (●) and nerve cell bodies (○) in the rat jejunum. SG, submucous ganglia; cm, circular muscle; MG, myenteric ganglia; lm, longitudinal muscle.



Fig. 6. Schematic outline of the topography and relative density of peptide-containing nerve fibers ($\bullet$) and nerve cell bodies ($\circ$) in the rat colon. SG, submucous ganglia; cm, circular muscle; MG, myenteric ganglia; lm, longitudinal muscle.

	Myenteric neurons		Submucous neurons	
	SI	LI	SI	LI
CGRP	↔ (2mm)	→ (5mm)	← (8mm)	↔ (6mm)
Enkephalin	← (7mm)	← (5mm)	0	0
Galanin	→ (15mm)	→ (6mm)	↔ (2mm)	↑
GRP	→ (20mm)	→ (10mm)	↔ (2mm)	↔ (3mm)
Somatostatin	→ (6mm)	ne	↔ (5mm)	ne
SP	→ (7mm)	← (5mm)	↔ (4mm)	ne
VIP	0	→ (3mm)	0	← (2mm)
VIP/NPY	→ (2mm)	→ (4mm)	← (4mm)	↑

Fig. 7. Polarities (marked with arrows) and projection distances (in mm) of various peptide-containing neuronal populations in the rat small (SI) and large (LI) intestine. → indicates descending projections, ← indicates ascending projections and ↑ indicates that no oro-anal projections can be demonstrated, 0 means absence of nerve fibers, ne stands for not examined.

Reinnervation: Return of GRP nerve fibers after local removal of myenteric ganglia (Paper V)

Myectomy of the jejunum resulted in the disappearance of GRP fibers from the underlying circular muscle and from myenteric ganglia and smooth muscle for approximately 10 mm anally to the lesion (see also paper I). As examined at different time intervals up to 60 weeks postoperatively, fine-varicose GRP fibers gradually reinnervated the portion anally to the lesion beginning at 20 weeks, first in the more disal portions and then (after 40-60 weeks) also in the more proximally located portions. Also the circular muscle of the myectomized segment became reinnervated during this period. At the 40-60 weeks stage the fibers were more numerous than in unoperated controls. In addition they were notably coarse and arranged in thick bundles. These findings indicate hyperinnervation of the myectomized circular muscle.

CONCLUDING REMARKS

Neuropeptides are beginning to gain acceptance as candidate neurotransmitters since they fulfill many of the criteria listed for such substances (for a review see Orrego 1979). Immunocytochemistry has proved to be a useful tool for the localization of a great many neuropeptides and for the demonstration of their coexistence. The coexistence of neuropeptides may be anticipated (when the peptides arise from the same precursor) or unexpected (when the peptides arise from different precursors) (c.f. Sundler et al 1985b). Anticipated coexistence is exemplified by VIP/PHI (Yanaihara et al 1983, Ekblad et al 1984) and SP/NKA (Sundler et al 1985a). However, coexistence has been demonstrated also for VIP/NPY (Ekblad et al 1984), SP/CGRP (Sundler et al 1985a), VIP/galanin (Melander et al 1985) and NPY/CGRP/CCK/Som (Furness et al 1985). The steadily growing list of peptide and peptide/conventional transmitter coexistence indicates that many (perhaps all) enteric neurons represent multimesenger systems.

There is mounting evidence that the constellation of coexisting peptides in a given neuronal system may vary among species. Thus, in the rat small intestine a population of submucous NPY neurons harbours in addition VIP whereas in the guinea pig the submucous NPY-containing neurons harbour CGRP, Som and CCK (Ekblad et al 1984, Furness et al 1985). In addition, dorsal root ganglia of the cat harbour constellations of coexisting peptides not found in for instance the rat (Leah et al 1985, Grunditz et al 1987). Further, the expression of neuropeptides and thereby also the constellation of coexisting peptides may vary during ontogeny (Ayer-LeLievre and Seiger 1984) and during different experimental conditions (Kessler et al 1983, Kiss et al 1984, Sawchenko et al 1984). Thus, it is by no means certain that the pattern of coexisting peptides

described here for the adult rat intestine will be found in other species or even in the rat during ontogeny and under pathophysiological conditions. The polarities of the intramural nerve fibers in the gut can be established by examining the axonal degeneration following local severing of nervous pathways. This has revealed characteristic projection patterns for peptide-containing neurons in the small and large intestine. On the average, the neuronal projections in the large intestine are shorter than in the small intestine suggesting that local reflex arcs in the large intestine are shorter. This may perhaps explain the difference in transit time between the colon and the small intestine. The precise mapping of each enteric nerve population with respect to distribution, polarity and projection distance will provide the necessary morphological basis for the understanding of the neuronal circuits involved in the regulation of gut motility as well as absorption and secretion. The studies on the long-term effects of myectomy have revealed a remarkable plasticity of enteric GRP neurons in that they display capacity for reinnervation and even hyperinnervation. The functional significance of the plasticity of enteric neurons can only be speculated upon. It may be associated with for instance the phenomenon of intestinal adaptation occurring after intestinal resection.

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References

- Alumets, J., Sundler, F. and Håkanson, R. (1977) Distribution, ontogeny and ultrastructure of somatostatin immunoreactive cells in the pancreas and gut. *Cell Tissue Res.* 185:465-479
- Alumets, J., Håkanson, R., Sundler, F. and Chang, K.J. (1978) Leu-enkephalin-like material in nerves and enterochromaffin cells in the gut. *Histochemistry* 56:187-196.
- Alumets, J., Fahrenkrug, J., Håkanson, R., Schaffalitzkay de Muckadell, O., Sundler, F. and Uddman, R. (1979) A rich VIP nerve supply is characteristic of sphincters. *Nature* 280:155-156.
- Amara, S.G., Jonas, V., Rosenfeld, M.G., Ong, E.S. and Evans, R.M. (1982) Alternative RNA processing in calcitonin gene expression generates mRNAs encoding different polypeptide products. *Nature* 298:240-244.
- Anagnostides, A.A., Christofides, N.D., Tatemoto, K., Chadwick, V.S. and Bloom, S.R. (1984) Peptide histidine isoleucine: a secretagogue in human jejunum. *Gut* 25:381-385.
- Anastasi, A., Erspamer, V. and Bucci, V. (1971) Isolation and structure of bombesin and alytesin, two analogous active peptides from skin of the European amphibians Bombina and Alytes. *Experientia* 27:166-167.
- Ayer-LeLievre, C. and Seiger, A. (1984) Parasympathetic neurons of salivary gland ganglia in fetal and postnatal rat are substance P-like immunoreactive. *Neurosci. Lett.* 51:287-292.
- Baetge, G. and Gershon, M.D. (1986) GABA in the PNS: Demonstration in enteric neurons. *Brain Res. Bull.* 16:421-424.
- Barthó, L. and Holzer, P. (1985) Search for a physiological role of substance P in gastrointestinal motility. *Neuroscience* 16:1-32.
- Baumgarten, H.G., Holstein, A.-F. and Owman, Ch. (1970) Auerbach's plexus of mammals and man: electron microscopic identification of three different types of neuronal processes in myenteric ganglia of the large intestine from Rhesus monkeys, guinea-pigs and man. *Z. Zellforsch. Mikrosk. Anat.* 106:376-397.
- Bertaccini, G., Impicciatore, M., Molina, E. and Zappia, L. (1974) Action of bombesin on human gastrointestinal motility. *Rend. Gastroenterol.* 6:45-51.
- Bitar, K.N. and Makhoulouf, G.M. (1982) Specific opiate receptors on isolated mammalian gastric smooth muscle cells. *Nature* 297:72-74.
- Brain, S.D., Williams, T.J., Tippins, H.R. and MacIntyre, I. (1985) Calcitonin gene-related peptide is a potent vasodilator. *Nature* 313:54-56.
- Brazeau, P., Vale, W., Burgus, R., Ling, N., Butcher, M., Rivier, J. and Guillemin, R. (1973) Hypothalamic polypeptide that inhibit the secretion of immunoreactive pituitary growth hormone. *Science* 179:77-79.
- Brennan, L.J., McLoughlin, T.A., Mutt, V., Tatemoto, K. and Wood, J.R. (1982) Effects of PHI, a newly isolated peptide, on gall-bladder function in the guinea-pig. *J. Physiol. (London)* 329:71-72.

- Brownstein, M.J. (1985) Peptide processing: An overview. In: Biogenetics of neurohormonal peptides. (eds. Håkanson, R. and Thorell, J.) Acad. Press, London. pp. 105-111.
- Christophe, J. and Robberecht, P. (1982) Effect of VIP on the exocrine pancreas. In: Vasoactive Intestinal Polypeptide (ed. S. Said) Raven Press, New York, pp. 235-252.
- Clague, J.R., Sternini, C. and Brecha, N.C. (1985) Localization of calcitonin gene-related peptide-like immunoreactivity in neurons of the rat gastrointestinal tract. *Neurosci. Lett.* 56:63-68.
- Cook, R.D. and Burnstock, G. (1976) The ultrastructure of Auerbach's plexus in the guinea-pig. I. Neuronal elements. *J. Neurocytol.* 5:171-194.
- Cooke, H.J. (1986) Neurobiology of the intestinal mucosa. *Gastroenterology* 90:1057-81.
- Coons, A.H., Leduc, E.H. and Connolly, J.M. (1955) Studies on antibody production. I. A method for the histochemical demonstration of specific antibody and its application to a study of the hyperimmune rabbit. *J. Exp. Med.* 102:49-60.
- Costa, M. and Furness, J.B. (1982) Nervous control of intestinal motility. In: Handbook of Experimental Pharmacology (ed. G. Bertaccini) Springer-Verlag, Berlin, Germany, 59:279-282.
- Costa, M., Furness, J.B., Cuello, A.C., Verhofstad, A.A.J., Steinbusch, H.W.M. and Elde, R.P. (1982) Neurons with 5-hydroxytryptamine-like immunoreactivity in the enteric nervous system: their visualization and reactions to drug treatment. *Neuroscience* 7:351-363.
- Costa, M. and Furness, J.B. (1983) The origins, pathways and terminations of neurons with VIP-like immunoreactivity in the guinea-pig small intestine. *Neurosci.* 8:665-676.
- Costa, M., Furness, J.B. and Cuello, A.C. (1985) Separate populations of opioid containing neurons in the guinea-pig intestine. *Neuropeptides* 5:445-448.
- Coupland, R.E. and Holmes, R.L. (1957) The use of cholinesterase techniques for the demonstration of peripheral nervous structures. *Quart. J. Microscop. Sci.* 98:327-330.
- Dogiel, A.S. (1895) Zur Frage ueber die Ganglien der Darmgeflechte bei den Säugetieren. *Anat. Anz.* 10:517-528.
- Dogiel, A.S. (1896) Zwei Arten sympatischer Nervenzellen. *Anat. Anz.* 11:679-687.
- Dogiel, A.S. (1899) Ueber den Bau der Ganglien in den Geflechten des Darms unter der Gallenblase des Menschen und der Säugetiere. *Arch. Anat. Physiol. Anat. Abt.* 130-158.
- Ekblad, E., Edvinsson, L., Wahlestedt, C., Uddman, R., Håkanson, R. and Sundler, F. (1984) Neuropeptide Y co-exists and co-operates with noradrenaline in perivascular nerve fibers. *Regul. Pept.* 8:225-235.

- Ekblad, E., Håkanson, R. and Sundler, F. (1984) VIP and PHI coexist with an NPY-like peptide in intramural neurones of the small intestine. *Regul. Pept.* 10:47-55.
- Ekblad, E., Håkanson, R., Sundler, F. and Wahlestedt, C. (1985) Galanin: Neuromodulatory and direct contractile effects on smooth muscle preparations. *Brit. J. Pharmacol.* 86:241-246.
- Elde, R., Hökfelt, T., Johansson, O. and Terenius, L. (1976) Immunohistochemical studies using antibodies to leucine-enkephalin: initial observations on the nervous system of the rat. *Neuroscience* 1:249-351.
- Erspamer, V. and Melchiorri, P. (1975) Actions of bombesin on secretion and motility of the gastrointestinal tract. In: *Gastrointestinal Hormones* (ed. J.C. Thompson) University of Texas Press, Austin, pp. 575-589.
- Euler von, U.S. and Gaddum, J.H. (1931) An unidentified depressor substance in certain tissue extracts. *J. Physiol. (London)* 72:74-87.
- Furness, J.B. and Costa, M. (1974) The adrenergic innervation of the gastrointestinal tract. *Ergebn. Physiol.* 69:1-51.
- Furness, J.B. and Costa, M. (1979) Actions of somatostatin on excitatory and inhibitory nerves in the intestine. *Eur. J. Pharmacol.* 55:69-74.
- Furness, J.B. and Costa, M. (1980) Types of nerves in the enteric nervous system. *Neuroscience* 5:1-20.
- Furness, J.B., Costa, M., Llewellyn-Smith, I.J., Franco, R. and Wilson, A.J. (1981) Polarity and projections of peptide-containing neurons in the guinea-pig small intestine. (eds. M.I. Grossman, M.A.B. Brazier and J. Lechago) Academic Press, New York.
- Furness, J.B. and Costa, M. (1982) Identification of gastrointestinal neurotransmitters. In: *Handbook of Experimental Pharmacology* (ed. G. Bertaccini) Springer-Verlag, Berlin, Germany, 59:383-460.
- Furness, J.B., Costa, M. and Eckenstein, F. (1983) Neurons localized with antibodies against choline acetyltransferase in the enteric nervous system. *Neurosci. Lett.* 40:105-109.
- Furness, J.B., Costa, M., Gibbins, I.L., Llewellyn-Smith, I.J. and Oliver, F.R. (1985) Neurochemically similar myenteric and submucous neurons directly traced to the mucosa of the small intestine. *Cell Tissue Res.* 241:155-163.
- Gabella, G. (1972) Fine structure of the myenteric plexus in the guinea-pig ileum. *J. Anat.* 111:69-97.
- Gabella, G. (1979) Innervation of the gastrointestinal tract. *Int. Rev. Cytol.* 59:129-193.
- Gershon, M.D. (1981) The enteric nervous system. *Ann. Rev. Neurosci.* 4:227-272.
- Ghatei, M.A., Christofides, N.D., Bishop, A.E., Mulderry, P.K., Bennet, A., Polak, J.M. and Bloom, S.R. (1984) Distribution and effect of calcitonin gene-related peptide in the gastrointestinal tract of the guinea-pig. *Regul. Pept.* 9:330 (A).

- Ghiglionne, M., Christofides, N., Yiangou, Y., Uttenthal, L.O. and Bloom, S.R. (1982) PHI stimulates intestinal fluid secretion. *Neuropeptides* 3:79-82.
- Gibbins, I.L., Furness, J.B., Costa, M., MacIntyre, I., Hillyard, C.J. and Girgis, S. (1985) Co-localization of calcitonin gene-related peptide-like immunoreactivity with substance P in cutaneous, vascular and visceral sensory neurons of guinea pigs. *Neurosci. Lett.* 57:125-130.
- Gintzler, A.R., Jaffe, B.M. and Baron, S.A. (1985) Dynamics of substance P neuronal transmission in the guinea pig enteric nervous system. In: *Tachykinin Antagonists. Proc. Fernström Symp.* (eds. Håkanson, R. and Sundler, F.) Elsevier, Amsterdam. Vol 6 pp. 213-223.
- Giraud, A., Jönsson, A.-C. and Dockray, G.J. (1984) Pro-enkephalin gene derived peptides in the porcine stomach: Cellular distribution and molecular forms. *Peptides* 5:757-763.
- Grunditz, T., Håkanson, R., Sundler, F. and Uddman, R. (1987) Neuronal pathways to the rat thyroid revealed by retrograde tracing and immunocytochemistry. *Neuroscience*, submitted.
- Guillemin, R. (1976) Somatostatin inhibits the release of acetylcholine induced electrically in the myenteric plexus. *Endocrinology* 99:1653-1654.
- Håkanson, R. and Sundler, F. (1979) Peptidergic system of the gastrointestinal tract. *Verh. Deutsch. Gesellsch. Inn. Med.* 85:1525-1534.
- Håkanson, R., Hedenbro, J., Liedberg, G. and Vallgren, S. (1982) Effect of vagotomy on gastric acid secretion in the rat. *Acta Physiol. Scand.* 115:135-139.
- Håkanson, R. and Sundler, F. (1983) The design of the neuroendocrine system: a unifying concept and its consequences. *Trends in Pharmacol. Sci.* 4:41-44.
- Hellström, P.M. (1985) Pharmacological analysis of the mechanisms of action for colonic contraction induced by neurotensin, substance P and methionine-enkephalin. *Acta Physiol. Scand* 125:13-24.
- Hirst, G.D.S., Holman, M.E. and Spence, I. (1974) Two types of neurons in the myenteric plexus of duodenum in the guinea pig. *J. Physiol. (London)* 231:303-326.
- Hökfelt, T., Efendic, S., Hellerström, C., Johansson, O., Luft, R. and Arimura, A. (1975) Cellular localization of somatostatin in endocrine-like cells and neurons of the rat with special reference to the A₁-cells of the pancreatic islets and to the hypothalamus. *Acta Endocr. (Kbh) Suppl.* 1-41.
- Hughes, J., Smith, T.W., Kosterlitz, H.M., Fothergill, L.A., Morgan, B.A. and Morris, R. (1975) Identification of two related pentapeptides from the brain with potent opiate activity. *Nature* 258:577-579.
- Itoh, N., Obata, K., Yanaihara, N. and Okamoto, H. (1983) Human preprovaso-active intestinal polypeptide contains a novel PHI-27-like peptide, PHM-27. *Nature* 304:547-549.

Jessen, K.R., Mirsky, R. and Hills, J.M. (1986) GABAergic neurons in the vertebrate peripheral nervous system. In: GABAergic Mechanisms in the Mammalian Periphery (eds. Erdö, S.L. and Bowery, N.G.) Raven Press, New York, pp. 117-134.

Kachelhoffer, J., Mendel, C., Dauchel, J., Hohmutter, D. and Grenier, J.F. (1976) The effects of VIP on intestinal motility. Study on in vivo perfused isolated canine jejunal loops. *Dig. Dis.* 21:957-962.

Kalbasi, H., Hudson, F.R., Herring, A., Moss, S., Glass, H.I. and Spencer, J. (1975) Gastric emptying following vagotomy and antrectomy and proximal gastric vagotomy. *Gut* 16:501-513.

Kessler, J.A., Adler, J.E., Bell, W.O. and Black, I.B. (1983) Substance P and somatostatin metabolism in sympathetic and special sensory ganglia in vitro. *Neuroscience* 9:309-318.

Kiss, J.Z., Mezey, E. and Skirboll, L. (1984) Corticotropin-releasing factor-immunoreactive neurons of the paraventricular nucleus became vasopressin positive after adrenalectomy. *Proc. Natl. Acad. Sci. USA* 81:1854-1858.

Kobayashi, S., Suzuki, M., Uchida, T. and Yanaihara, N. (1984) Enkephalin neurons in the guinea pig duodenum: A light and electron microscopic immunocytochemical study using an antiserum to methionine-enkephalin-arg⁶-gly⁷-leu⁸. *Biomed. Res.* 5:489-506.

Kosterlitz, H.W. and Watt, A.J. (1975) The peristaltic reflex. In: *Methods in Pharmacology* (eds. E.E. Daniel and D.M. Paton) Plenum, New York, 3:391-401.

Larsson, L.-I. (1981) Peptide immunocytochemistry. *Prog. Histochem. Cytochem.* 13:1-85.

Leah, J.D., Cameron, A.A., Kelly, W.L. and Snow, P.J. (1985) Coexistence of peptide immunoreactivity in sensory neurons of the cat. *Neuroscience* 16:683-690.

Leander, S., Håkanson, R., Rosell, S., Folkers, K., Sundler, F. and Tornqvist, K. (1981) A specific substance P antagonist blocks smooth muscle contractions induced by non-cholinergic, non-adrenergic nerve stimulation. *Nature* 294:467-469.

Legay, C., Saffrey, M.J. and Burnstock, G. (1984) Coexistence of immunoreactive substance P and serotonin in neurones of the gut. *Brain Res.* 302:379-382.

Lembeck, F. (1953) Zur Frage der zentralen Übertragung afferenter Impulse. III. Mitteilung. Das Vorkommen und die Bedeutung der Substanz P in der dorsalen Wurzeln des Rückenmarks. *Arch. Exp. Pathol. Pharmac.* 219:193-213.

Llewellyn-Smith, I.J., Furness, J.B., Wilson, A.J. and Costa, M. (1983) Organization and fine structure of enteric ganglia. In: *Autonomic ganglia* (ed. L.G. Elfvig) John Wiley & Sons Ltd. Chichester, Great Britain, pp. 145-182.

Makhlof, G.M. (1985) Enteric neuropeptides: role in neuromuscular activity of the gut. *Trends in Pharm. Sci* 6:214-218.

Malmfors, G., Leander, S., Brodin, E., Håkanson, R., Holmin, T. and Sundler, F. (1981) Peptide containing neurones intrinsic to the gut wall. An experimental study in the pig. *Cell Tissue Res.* 214:225-238.

- McDonald, T.J., Nilsson, G., Vagne, M., Ghatei, M., Bloom, S.R. and Mutt, V. (1978) A gastrin releasing peptide from non-antral gastric tissue. *Gut* 19:767-774.
- Melander, T., Hökfelt, T., Rökaeus, Å., Fahrenkrug, J., Tatemoto, K. and Mutt, V. (1985) Distribution of galanin-like immunoreactivity in the gastrointestinal tract of several mammalian species. *Cell Tissue Res.* 239:253-270.
- Moghimzadeh, E., Ekman, R., Håkanson, R., Yanaihara, N. and Sundler, F. (1983) Neuronal gastrin-releasing peptide in the mammalian gut and pancreas. *Neuroscience* 10:553-563.
- Mukhopadhyay, A.K. and Weisbrodt, N.W. (1975) Neural organization of esophageal peristalsis: Role of vagus nerve. *Gastroenterology* 68:444-447.
- Nishi, S. and North, R.A. (1973) Intracellular recording from the myenteric plexus of the guinea pig ileum. *J. Physiol (London)* 231:471-491.
- Norberg, K.A. (1964) Adrenergic innervation of the intestinal wall studied by fluorescence microscopy. *Int. J. Neuropharmacol.* 3:379-382.
- Nawa, H., Hirose, T., Takashima, H., Inayama, S. and Nakanishi, S. (1983) Nucleotide sequences of cloned cDNA's for two types of bovine brain substance P precursor. *Nature* 306:31-36.
- Nilsson, G., Larsson, L.-I., Håkanson, R., Brodin, E., Pernow, B. and Sundler, F. (1975) Localization of substance P-like immunoreactivity in mouse gut. *Histochemistry* 43:97-99.
- Numa, S. (1985) Precursors of opioid peptides and corticotropin-releasing factor and their genes. In: *Biogenetics of neurohormonal peptides* (eds. R. Håkanson and J. Thorell) Acad. Press Inc. London, pp. 29-46.
- Ohkubo, K. (1936) Studies on the intrinsic nervous system of the digestive tract. I. The sub-mucous plexus of guinea-pig. *Japan. J. Med. Sci.* 6:1-20.
- Oki, M. and Daniel, E.E. (1977) Effects of vagotomy on the ultrastructure of the of dog stomach. *Gastroenterology* 73:1029-1040.
- Orrego, F. (1979) Criteria for the identification of central neurotransmitters, and their application to studies with some nerve tissue preparations in vitro. *Neuroscience* 4:1037-1057.
- Otsuka, M., Konishi, S. and Takahashi, T. (1975) Hypothalamic substance P as a candidate for transmitter of primary afferent neurons. *Fed. Proc.* 34:1922-1928.
- Otsuka, M. and Konishi, S. (1983) Substance P - the first peptide neurotransmitter? *Trends in NeuroSci.* 6:317-320.
- Pernow, B. (1983) Substance P. *Pharmac. Rev.* 35:85-141.
- Polak, J.M. and Bloom, S.R. (1978) Peptidergic nerves in the gastrointestinal tract. *Invest. Cell Pathol.* 1:301-326.
- Polak, J.M. and Bloom, S.R. (1982) Distribution and tissue localization of VIP in the central nervous system and in seven peripheral organs. In: *Vasoactive intestinal polypeptide* (ed. S. Said) Raven Press, New York, pp. 107-120.

- Raptis, S., Schleger, W. and Pfeiffer, E.F. (1978) Effects of somatostatin on gut and pancreas. In: Gut Hormones (ed. Bloom, S.R.) Churchill Livingstone, London, pp. 446-452.
- Rosenfeld, M.G., Mermod, J.J., Amara, S.G., Swanson, L.W. and Evans, R.M. (1983) Production of a novel neuropeptide encoded by the calcitonin gene via tissue specific RNA processing. *Nature* 304:129-135.
- Rökæus, Å., Melander, T., Hökfelt, T., Lundberg, J.M., Tatemoto, K., Carlquist, M. and Mutt, V. (1984) A galanin-like peptide in the central nervous system and intestine of the rat. *Neurosci. Lett.* 47:161-166.
- Said, S.I. and Mutt, V. (1970) Polypeptide with broad biological activity: isolation from small intestine. *Science* 169:1217-1218.
- Said, S.I. (1973) Smooth-muscle relaxant activity of vasoactive intestinal polypeptide. In: *Endocrinology 1973, Proc. 4th Int. Symposium*, London, pp. 297-301.
- Said, S.I. (1982) Vasodilator action of VIP: Introduction and general considerations. In: *Vasoactive intestinal polypeptide* (ed. S. Said) Raven Press New York, pp. 145-148.
- Sauer, M.E. and Rumble, C.T. (1946) The number of nerve cells in the myenteric and submucous plexus of the small intestine of the cat. *Anat. Record* 96:373-381.
- Sawchenko, P.E., Swanson, L. and Vale, W.W. (1984) Co-expression of corticotropin-releasing factor and vasopressin immunoreactivity in parvocellular neurosecretory neurons of the adrenalectomized rat. *Proc. Natl. Acad. Sci. USA* 81:1883-1887.
- Schofield, G.C. (1968) Anatomy of muscular and neuronal tissues in the alimentary canal. In: *Handbook of Physiology. Vol IV, The Alimentary canal* (ed. C.F. Code) Maryland, Waverly Press, pp 1579-1628.
- Schultzberg, M., Hökfelt, T., Nilsson, G., Terenius, L., Rehfeld, F.F., Brown, M., Elde, R., Goldstein, M. and Said, S. (1980) Distribution of peptide- and catecholamine-containing neurons in the gastrointestinal tract of rat and guinea-pig: immunohistochemical studies with antisera to substance P, vasoactive intestinal polypeptide, enkephalins, somatostatin, gastrin/cholecystokinin, neurotensin and dopamine β -hydroxylase. *Neuroscience* 5:689-744.
- Schultzberg, M. and Dalsgaard, C.-J. (1983) Enteric origin of bombesin immunoreactive fibers in the rat coeliac-superior mesenteric ganglion. *Brain Res.* 269:190-195.
- Stach, W. (1982) Zur neuronalen Organisation des Plexus Myentericus (Auerbach) im Schweindünndarm. VI Typ IV-Neurone. *Z. mikrosk. Anat. Forsch.* 6:972-994.
- Stjernquist, M., Ekblad, E., Owman, Ch. and Sundler, F. (1986) Neuronal localization and motor effects of gastrin-releasing peptide (GRP) in rat uterus. *Regul. Pept.* 13:197-205.

Sundler, F., Håkanson, R., Larsson, L.-I., Brodin, E. and Nilsson, G. (1977) Substance P in the gut: An immunochemical and immunohistochemical study of its distribution and development. In: Substance P (eds. von Euler, U.S. and Pernow, B) Raven Press, New York, pp. 59-65.

Sundler, F., Alumets, J. and Håkanson, R. (1978) Peptides in the gut with a dual distribution in nerves and endocrine cells. In: Gut Hormones (ed. Bloom, S.R.) Churchill, Livingstone London, pp. 406-413.

Sundler, F., Håkanson, R. and Leander, S. (1980) Peptidergic nervous systems in the gut. Clin. Gastroenterol. 9:517-543.

Sundler, F., Håkanson, R., Leander, S. and Uddman, R. (1982) Neuropeptides in the gut wall: Cellular and subcellular localization, topographic distribution and possible physiological significance. In: Cytochemical Methods in Neuroanatomy. (eds. S. Palay and V. Chan-Palay) Alan R. Liss, Inc., New York, pp. 341-356.

Sundler, F., Brodin, E., Ekblad, E., Håkanson, R. and Uddman, R. (1985a) Sensory nerve fibers: Distribution of substance P, neurokinin A and calcitonin gene-related peptide. In: Tachykinin Antagonists. Proc. Fernström Symp. (eds. R. Håkanson and F. Sundler) Elsevier, Amsterdam. Vol. 6. pp. 3-14.

Sundler, F., Ekblad, E., Böttcher, G., Alumets, J. and Håkanson, R. (1985b) Coexistence of peptides in the neuroendocrine system. In: Biogenetics of Neurohormonal Peptides. (eds. R. Håkanson and J. Thorell) Acad Press, London, pp. 213-243.

Tatemoto, K., Rökaeus, Å., Jörnwall, H., MacDonald, T.J. and Mutt, V. (1983) Galanin - a novel biologically active peptide from porcine intestine. FEBS Lett. 164:124-129.

Tippins, J.R., Morris, H.R., Panico, M., Etienne, T., Bevis, P., Birgis, S., MacIntyre, I., Azria, M. and Attinger, M. (1984) The myotropic and plasma-calcium modulating effects of calcitonin gene-related peptide. Neuropeptides 4:425-434.

Tramu, G., Pillez, A. and Leonardelli, J. (1980) An efficient method of antibody elution for the successive or simultaneous location of two antigens by immunocytochemistry. J. Histochem. Cytochem. 26:322-324.

Uddman, R., Moghimzadeh, E. and Sundler, F. (1984) Occurrence and distribution of GRP-immunoreactive nerve fibers in the respiratory tract. Arch. Oto-rhinolaryngol. 239:145-151.

Van Driel, C. and Drukker, J. (1973) A contribution to the study of the architecture of the autonomic nervous system of the digestive tract of the rat. J. Neural Transm. 34:301-320.

Van Neuten, J.M., Van Ree, J.M. and Vanhoutte, P.M. (1977) Inhibition by met-enkephalin of peristaltic activity in the guinea pig ileum, and its reversal by naloxone. Eur. J. Pharmacol. 41:341-342.

Vincent, S.R., Dalsgaard, C.-J., Schultzberg, M., Hökfelt, T., Christensson, I. and Terenius, L. (1984) Dynorphin-immunoreactive neurons in the autonomic nervous system. Neuroscience 11:973-987.

Wang, Y.-N. and Lindberg, I. (1986) Distribution and characterization of the opioid octapeptide met⁵-enkephalin-arg⁶-gly⁷-leu⁸ in the gastrointestinal tract of the rat. *Cell Tissue Res.* 244:77-85.

Waterfield, A.A., Smokcum, R.W.J., Hughes, J., Kosterlitz, H.W. and Henderson, G. (1977) In vitro pharmacology of the opioid peptides, enkephalins and endorphins. *Eur. J. Pharmacol.* 43:107-116.

Wood, J.D. (1983) Neurophysiology of parasympathetic and enteric ganglia. In: *Autonomic ganglia* (ed. L.G. Elfvig) John Wiley & Sons Ltd, Chichester, Great Britain, pp. 367-398.

Yanaihara, N., Nokiya, K., Yanaihara, C., Iwanaga, T. and Fujita, T. (1983) Immunocytochemical demonstration of PHI and its co-existence with VIP in intestinal nerves of the rat and pig. *Arch. Histol. Jap.* 46:575-581.

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GRP neurones in the rat small intestine issue long anal projections

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Summary

Gastrin releasing peptide (GRP) immunoreactive nerve fibres are numerous in the gut wall. Nerve cell bodies containing GRP are regularly found in the myenteric ganglia. The projections of GRP neurones in the rat small intestine were studied after myectomy or transection of the gut wall. Operated rats were left for 8–10 days or 5 weeks. Specimens were studied by immunocytochemistry, immunochemistry and in vitro for motor activity. GRP fibres were absent and GRP was markedly reduced in the gut wall underlying the area of myectomy and 10 mm anally to the myectomy or site of transection. Further anally, GRP and the GRP fibres gradually returned and were back to normal 25–30 mm from the lesion. Myenteric GRP neurones in the rat small intestine thus project anally over a distance of approximately 20–25 mm. A series of experiments was performed in order to test the idea that GRP is directly involved in intestinal motor functions. The results did not support this view. Strips of longitudinal smooth muscle with adherent myenteric ganglia were taken orally and anally to the myectomy and the motor activity of the specimens was compared. Electrical stimulation evoked a contractile response in the oral segment that was 6 times greater than that of the anal segment. However, GRP (10^{-9} – 10^{-5} M) did not evoke contraction and the electrically induced contractile response was unaffected by GRP but could be blocked by atropine. The reduced contractile response in the 'denervated' anal segment is thus probably not due to a shortage of GRP fibres.

neuropeptides; gastrin releasing peptide, GRP; myenteric neurones; neuronal projections; gut innervation

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Introduction

Peptide-containing neurones are prominent in the gut. Among recognized gut neuropeptides are substance P, vasoactive intestinal peptide (VIP), enkephalin, somatostatin and gastrin-releasing peptide (GRP) [1-3]. GRP-storing fibres are numerous in the smooth muscle and myenteric ganglia. Nerve cell bodies containing GRP are regularly found in the myenteric ganglia [2,3].

The functional significance of the many peptide-containing neuronal systems in the gut is not fully understood. Several observations indicate that they participate in the regulation of gut motility. The assessment of their role in gut peristalsis would be facilitated by knowledge of the polarity and projections of each individual neuronal system. The projections of intramural gut neurones can be established by for example, myectomy, myotomy or complete transection of the gut because of the ensuing axonal degeneration. Thanks to the pioneering work of Furness and Costa and their colleagues, such knowledge is now becoming available [4-8]. The present report deals with the projections of the GRP immunoreactive neurones in the rat small intestine as studied by denervation procedures in combination with immunocytochemistry, immunochemistry and *in vitro* analysis of motor activity.

Materials and Methods

24 rats were subjected to small intestinal myectomy, i.e. circumferential removal of 10 mm of the outer longitudinal smooth muscle layer together with adherent myenteric ganglia at the mid-jejunal level as described by Furness and Costa [4,5]. 10 rats were subjected to transection of the entire gut wall (jejunum) and subsequent joining of the severed parts end-to-end. The animals were left for either 8-10 days or 5 weeks after either operation. They were killed by decapitation and two 5-cm segments were taken, one orally and one anally to the lesion (transection or myectomy). The tissue specimens were processed either for immunocytochemistry (myectomy 6 rats, intestinal transection 6 rats) or radioimmunochemistry (myectomy 12 rats, intestinal transection 4 rats) or used for *in vitro* studies (myectomy 6 rats). In addition, 5 control rats were used to study the distribution of GRP fibres in the mid-jejunum.

Immunocytochemistry

Each 5-cm segment from operated rats and corresponding segment from control rats was opened along the mesenteric border and pinned flat on a piece of balsa wood with the mucosal side facing the wood. The preparations were fixed by floating in a mixture of formaldehyde and picric acid (Stefanini's fixative) overnight, and thoroughly rinsed in buffer containing 10% sucrose. The outer (longitudinal) and inner (circular) muscle layers were separated, placed on chrome-alum coated slides and oriented in such a way that the oral and anal ends could be identified. Before further processing the whole mounts were dried, frozen and thawed. Alternatively, the 5-cm segments were opened and cut serially in 5-mm pieces. The specimens were

immersed overnight in 4% formaldehyde in 0.1 M phosphate buffer (pH 7.2) and thoroughly rinsed in sucrose-enriched buffer. They were frozen on dry ice and transverse sections were cut in a cryostat at 10–20 μm thickness. Sections and whole mounts were processed for the immunocytochemical demonstration of GRP using a previously characterized rabbit antiserum (code no. R-6902, kindly donated by Prof. N. Yanaihara, Shizuoka, Japan) [9]. The antiserum was used in dilution, 1:640.

The site of the antigen-antibody reaction was revealed by the indirect immunofluorescence method of Coons et al. [10]. For controls we used antiserum which had been inactivated by the addition of excess amounts of antigen (10–100 μg synthetic GRP per ml diluted antiserum). The antiserum was inactivated also by bombesin (Peninsula, Belmont, CA) (10–100 μg per ml diluted antiserum), confirming that the antiserum is directed against the C-terminal portion of GRP [9]. The antiserum did not cross-react with other known or putative neuropeptides, such as substance P, physalaemin, Leu- and Met-enkephalin, VIP, somatostatin, cholecystokinin octapeptide or neurotensin (100 μg per ml diluted antiserum). Nevertheless, cross-reactivity with other peptides or protein cannot be excluded. Therefore, it would be appropriate to refer to the immunoreactive material as GRP-like rather than GRP. For simplicity, the shorter term is used in the following.

Immunocytochemistry

Each segment was opened and rinsed and cut serially in 5-mm specimens, 4–5 orally and 6 anally to the lesion. The specimens were weighed and boiled in redistilled water (0.1 g wet weight/ml) for 2 min. Following the addition of 3 M acetic acid to a final concentration of 0.5 M, the tissue was homogenized (Polytron, 1–2 min), and extracted at room temperature for 30 min. After centrifugation at $2000 \times g$ for 15 min the supernatants were collected and lyophilized. For radioimmunoassay, the freeze-dried material was taken up in 2 ml of 0.05 M phosphate buffer (pH 7.4).

Details of the radioimmunoanalysis of GRP have been given elsewhere [3]. Briefly, bombesin antiserum (code no. 8050, Milab, Malmö) was used in dilution, 1:14000. Under the assay conditions this antiserum cross-reacts with GRP (30%) and GRP 14–27 (37%) suggesting that the antibodies recognize the C-terminal portion of bombesin which is shared by GRP. The concentrations were recalculated and expressed as pg GRP equivalents per g wet weight.

Motor activity studies

Myectomized rats were killed 8–10 days after the operation. Strip preparations, 15 mm in length, consisting of longitudinal smooth muscle and adherent myenteric ganglia, were dissected out in oral and anal directions from the lesion. The preparations were placed in a modified Krebs solution with the following composition (in mM): NaCl, 133; NaHCO_3 , 16.3; KCl, 4.7; MgCl_2 , 1.0; NaH_2PO_4 , 1.4; CaCl_2 , 2.5; and glucose, 7.8. The solution was bubbled with a gas mixture of 7% CO_2 in O_2 giving pH 7.2–7.3. The strip preparations were mounted vertically on a perspex holder in a 7-ml organ bath thermostated at 37°C. One end was attached to a rigid support and the free end to a lever connected via a spring to a Grass FT 03

force displacement transducer for isotonic registration of mechanical activity [2,11]. The load on the muscle was set at 0.2 g. Platinum ring electrodes were placed around the muscle strip with a constant electrode distance of 5 mm. The electrodes were connected to a Grass S4C stimulator for field stimulation with square wave pulses (10–15 V over the electrodes, 1 ms duration, frequency 1–10 Hz, pulse trains lasting for 3 s). The polarity of the electrodes was shifted after each stimulation period to avoid polarization. The mechanical activity was recorded continuously throughout the experiment on a Grass model 7 Polygraph. The preparations were allowed to equilibrate in the bath for 1 h before experimentation started.

Drugs

The following drugs were used: atropine sulphate from TACO, Stockholm; tetrodotoxin from Sankyo, Tokyo; synthetic GRP from Peninsula, Belmont, CA.

Results

Immunocytochemical findings

As studied in control rats, delicate, varicose GRP fibres were numerous in the smooth muscle layers and in the myenteric ganglia of the mid-jejunum; the mucosa

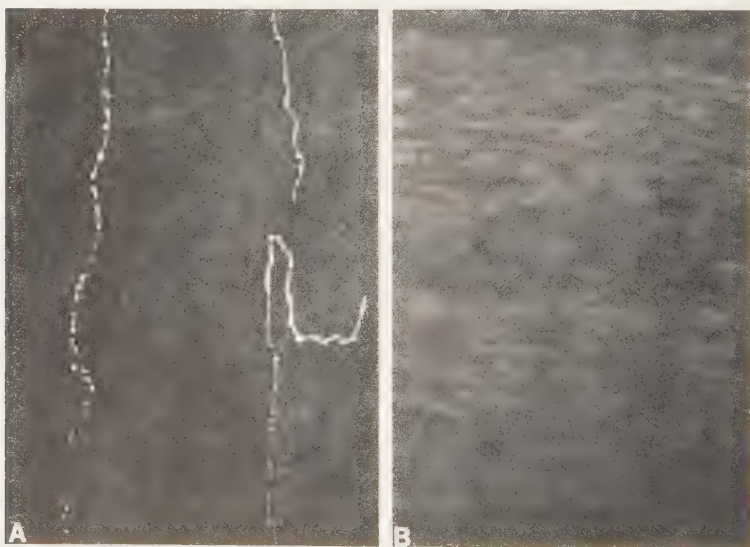


Fig. 1. Whole mounts of intestinal circular smooth muscle. (A) Control rat. Delicate fine varicose GRP immunoreactive nerve fibres running in between the muscle bundles. (B) Removal of longitudinal smooth muscle with adherent myenteric ganglia (myectomy) causes total disappearance of GRP fibres in the smooth muscle underlying the lesion as studied 10 days later. $\times 275$

harboured only few GRP fibres, predominantly in the basal portion. Moderately immunoreactive GRP nerve cell bodies occurred sparsely in the myenteric ganglia, none was found in the submucous ganglia. After myectomy the GRP fibres disappeared from the underlying, circular smooth muscle layer (Fig. 1) and from the mucosa and submucosa. Within a narrow area of approximately 1–2 mm orally to the myectomy or site of transection, the number of GRP fibres had decreased slightly in the longitudinal smooth muscle (Fig. 2). Anally to the myectomy or site of transection demonstrable GRP fibres disappeared from the gut wall for a distance of approximately 10 mm. Further distally to this point the GRP fibres returned gradually (Fig. 3A). Twenty-five to thirty mm distally to the lesion the density of GRP fibres was back to normal as judged from comparison with control rats (Fig.



Fig. 2. Whole mounts of longitudinal smooth muscle with adherent myenteric ganglia prepared from myectomized rat (10 days postoperatively). (A) GRP fibres are moderate in number, 2 mm orally from the lesion. Proximal to this point GRP fibres display the normal number and distribution. (B) GRP fibres are numerous 10 mm orally. The results were identical following transection of the gut $\times 200$

3B). Moderately GRP-immunoreactive nerve cell bodies were occasionally seen both orally and anally to the lesion. The loss of GRP nerve fibres was equally striking 8–10 days and 5 weeks after the operations.

Immunochemical findings

The concentrations of GRP in the different 5-mm specimens taken orally and anally to the lesions are shown in Fig. 4. The concentration was markedly reduced in the gut wall underlying the lesion in the whole wall up to 20 mm anally, 8–10 days postoperatively. Five weeks after myectomy the GRP concentration was reduced for 30 mm in the anal direction. In addition, the concentration of GRP seemed reduced in the first 5-mm oral segments after transection, but not after myectomy.

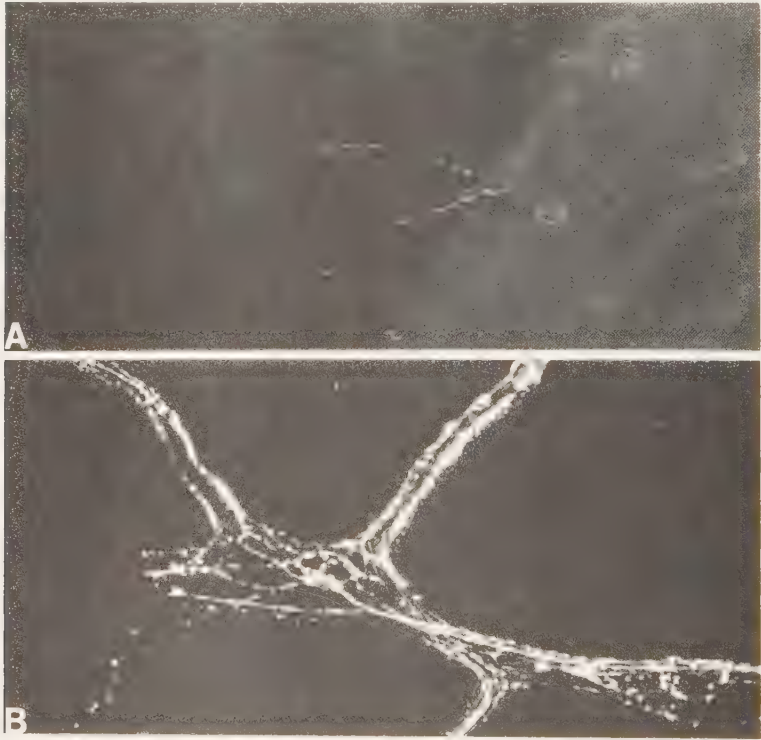


Fig. 3. Whole mounts of longitudinal smooth muscle with adherent myenteric ganglia from myectomized rat (10 days postoperatively). (A) GRP fibres are very few and weakly immunoreactive 10 mm anally to the lesion. (B) GRP fibres are numerous and more intensely fluorescent 25 mm anally to the lesion. Between these points the number of GRP fibres increased gradually. The results were identical following transection of the gut. $\times 200$.

In vitro motor activity

The smooth muscle preparations displayed no spontaneous activity. Electrical field stimulation evoked a frequency dependent contraction. The contraction was abolished by the muscarinic blocker atropine (10^{-6} M) (Fig. 5) and by tetrodotoxin (10^{-6} M), a blocker of nervous conduction. The amplitude of the contractile responses differed markedly between specimens taken orally or anally to the myectomy (Fig. 5). Upon a standardized electrical field stimulation (3 Hz, 1 ms, 10 V over the electrodes) for 3 s, the mean contractile response of the oral segments was 0.39 ± 0.07 mN ($n = 6$) whereas that of the corresponding anal segments was 0.07 ± 0.02 mN ($n = 6$) ($P < 0.001$). Addition of GRP (10^{-9} – 10^{-5} M) did not induce contraction, neither did it affect the electrically induced contractile response (Fig. 5).

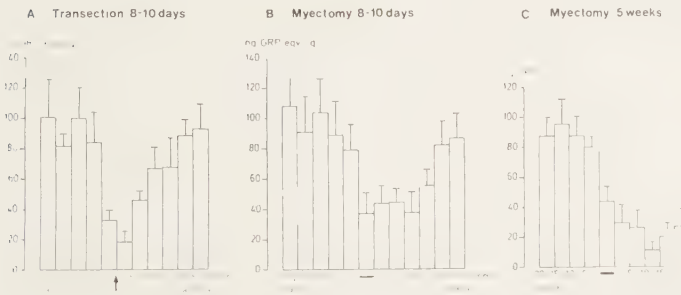


Fig. 4. GRP concentration profile orally and anally to the lesions. (A) Transection 8–10 days postoperatively ($n = 4$). (B) Myectomy 8–10 days postoperatively ($n = 4$ –7). (C) Myectomy 5 weeks postoperatively ($n = 5$). The concentrations are expressed as ng GRP equivalents per g wet weight. Values are means $\pm$ S.E.M. Horizontal line indicates the area of myectomy. Arrow indicates the site of transection.

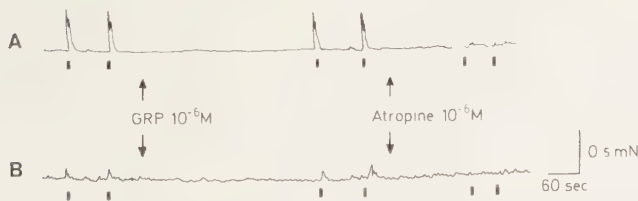


Fig. 5. Tracings of electrically induced contractions of isolated longitudinal muscle with adherent myenteric ganglia taken orally and anally to the myectomy. Electrical stimulation (3 Hz, 1 ms, 10 V, for 3 s) (black rectangles) evoked a contractile response. The contraction of the oral strip (A) was approximately 6 times stronger than that of the anal strip (B). GRP (10^{-6} M) had no contractile effect and did not affect the electrically induced contraction. Atropine (10^{-6} M) abolished the electrically induced contractions.

Discussion

GRP is a major gut neuropeptide, distributed in all layers of the gut wall but predominating in the smooth muscle and myenteric ganglia [2,3]. Available evidence indicates that the majority of GRP neurones are intrinsic to the gut wall. The results of the present study indicate that myenteric GRP neurones project anally for a distance of 20–25 mm and possibly also orally for a few millimeters. GRP cell bodies are found scattered in the myenteric plexus throughout the small intestine. The fact that demonstrable GRP fibers were lacking immediately distal to the lesion may reflect a low content of GRP in the preterminal portion of the fibers. Previous studies of the guinea pig small intestine have revealed several types of peptide-containing neurones with anal projections, such as those storing substance P, VIP, somatostatin, enkephalin [4] and NPY [7]. None of these types of neurones seem to have projections longer than 5 mm. Recently, Costa et al. [8] described the intramural distribution and projections of GRP neurones in the guinea pig small intestine and found these neurones to have long anal projections (approx. 15 mm). The present results indicate that GRP neurones in the small intestine of the rat also project over a notably long distance.

If neuronal GRP exerts a direct effect on intestinal smooth muscle, it is to be expected that the motor activity of control specimens of longitudinal smooth muscle should be different from that of GRP-depleted specimens. This proved to be the case, but we obtained no evidence that this reflected the loss of GRP. Electrical stimulation of specimens taken orally to the myectomy induced a strong contractile response which was blocked by atropine and thus cholinergic in nature. In specimens taken anally to the lesion the contractile response was weaker. GRP did not contract the longitudinal smooth muscle of the rat small intestine, unlike the situation in the guinea pig and some other animals [2,12,13]. Neither did it restore the contractile response in the anal, GRP-depleted segment. Thus, if neuronal GRP is at all involved in the contraction induced by electrical stimulation, the effect is indirect. It appears possible that the motor effects of the lesions reflect the functional significance of cholinergic rather than GRP fibres.

The long anal projections in the intestinal smooth muscle suggest that the GRP neurones are involved in peristalsis or peristaltic reflexes. The lack of motor effects of GRP on the longitudinal muscle of the rat small intestine makes the exact role of the GRP neurones enigmatic.

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References

- 1 Sundler, F., Håkanson, R. and Leander, S., Peptidergic nervous systems in the gut, *Clin. Gastroenterol.*, 9 (1980) 517–543

- 2 Leander, S., Ekman, R., Uddman, R., Sundler, F. and Hakanson, R., Neuronal cholecystokinin, gastrin-releasing peptide, neurotensin and β -endorphin in guinea pig intestine. Distribution and possible motor functions, *Cell Tissue Res.*, 235 (1984) 521-531.
- 3 Moghizadeh, E., Ekman, R., Hakanson, R., Yanaihara, N. and Sundler, F., Neuronal gastrin-releasing peptide in the mammalian gut and pancreas, *Neuroscience*, 10 (1983) 553-563.
- 4 Furness, J.B., Costa, M., Franco, R. and Llewellyn-Smith, I.J., Neuronal peptides in the intestine: distribution and possible functions. In M. Costa and M. Trabucchi (Eds.), *Neuronal Peptides and Neuronal Communication*, Raven Press, New York, 1980, pp. 601-617.
- 5 Costa, M., Furness, J.B., Llewellyn-Smith, I.J., Davies, B. and Oliver, J., An immunohistochemical study of the projections of somatostatin-containing neurones in the guinea pig intestine, *Neuroscience*, 5 (1980) 841-852.
- 6 Costa, M. and Furness, J.B., Neuronal peptides in the intestine, *Br. Med. Bull.*, 38 (1982) 247-252.
- 7 Furness, J.B., Costa, M., Emson, P.C., Hakanson, R., Moghizadeh, E., Sundler, F., Taylor, I.L. and Chance, R.E., Distribution, pathways and reactions to drug treatment of nerves with neuropeptide Y- and pancreatic polypeptide-like immunoreactivity in the guinea pig digestive tract, *Cell Tissue Res.*, 234 (1983) 71-92.
- 8 Costa, M., Furness, J.B., Yanaihara, N., Yanaihara, C. and Moody, T.W., Distribution and projections of neurones with immunoreactivity for both gastrin-releasing peptide and bombesin in the guinea pig small intestine, *Cell Tissue Res.*, 235 (1984) 285-293.
- 9 Yanaihara, N., Yanaihara, C., Mochizuki, T., Imura, K., Fujita, T. and Iwanaga, T., Immunoreactive GRP, *Peptides*, 2 (Suppl. 2) (1981) 185-192.
- 10 Coons, R.D., Leduc, E.H. and Connolly, J.M., Studies on antibody production. I. A method for the histochemical demonstration of specific antibody and its application to a study of the hyperimmune rabbit, *J. Exp. Med.*, 102 (1955) 49-60.
- 11 Ishida, Y. and Krakowa, N., Isometric and isotonic spontaneous contractions of guinea pig taenia coli, *Jap. J. Pharmacol.*, 24 (1974) 925-927.
- 12 Erspamer, V., Peptides of the amphibian skin active on the gut. II. Bombesin-like peptides: Isolation, structure and basic functions. In G.B.J. Glass (Ed.) *Gastro-intestinal Hormones*, Raven Press, New York, 1980, pp. 343-362.
- 13 Bertaccini, G., Peptides: Candidate hormones. In G. Bertaccini, (Ed.), *Handbook of Experimental Pharmacology*, Springer-Verlag, Berlin-Heidelberg New York, 1982, pp. 85-135.

GALANIN NERVE FIBERS IN THE RAT GUT: DISTRIBUTION, ORIGIN AND PROJECTIONS

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Abstract—Galanin, a 29 amino acid peptide, was recently isolated from the porcine gut. Immunocytochemistry revealed a dense network of galanin-immunoreactive nerve fibers in the submucosa, smooth muscle layers and intramural ganglia throughout the rat gastrointestinal tract. In the smooth muscle the density of innervation was lower in the colon than in the small intestine. In the mucosa galanin-immunoreactive fibers were quite numerous in the small intestine, less numerous in the large intestine and rare in the stomach. A moderate number of galanin-immunoreactive nerve cell bodies could be detected in the myenteric ganglia throughout the digestive tract and in the submucosal ganglia of the intestines. Enteric galanin-immunoreactive fibers seem to be intrinsic to the gastrointestinal tract since their distribution and frequency were unaffected after extrinsic denervation (chemical sympathectomy, vagal denervation or clamping of nerves running within the mesenterium). Myectomy at the mid-jejunal level resulted in a total loss of galanin-immunoreactive nerve fibers 5 mm anally to the lesion with a gradual return of galanin-immunoreactive fibers up to 15–20 mm further anally; there was no overt loss of fibers orally. The findings indicate that myenteric galanin-immunoreactive neurones issue long descending projections that terminate either within the myenteric ganglia or in the smooth muscle.

The digestive tract is rich in neuropeptides, probably playing important roles in secretory and motor activities.^{3,11,15,17} Among gut neuropeptides demonstrated immunochemically and immunocytochemically are: substance P, vasoactive intestinal polypeptide, somatostatin, enkephalin, neuropeptide Y and gastrin-releasing peptide (GRP).

Galanin, a 29 amino acid peptide with N-terminal glycine and C-terminal alanine amide, was recently isolated from the porcine upper small intestine.¹⁸ Galanin-immunoreactive nerve cell bodies and fibers have been demonstrated by immunocytochemistry in the gastrointestinal tract and brain of the rat.¹⁴ Radioimmunochemical analysis and chromatographic characterization have confirmed the presence of galanin-like material in extracts of the intestine and brain.¹⁴

The present study is concerned with the origin, distribution and projections of the galanin-immunoreactive nerve fibers in the rat gastrointestinal tract. The origin of the fibers was deduced from the results of various denervations: chemical sympathectomy (6-hydroxydopamine treatment), vagal denervation and clamping of extrinsic nerves within the mesenterium. The enteric projections were established by examination of the axonal degeneration following microsurgical lesions of intramural nerve pathways (myectomy). Such techniques have previously been used to examine projections of enteric neurones

storing substance P, vasoactive intestinal polypeptide, somatostatin, enkephalin,⁹ neuropeptide Y¹⁰ or GRP.

EXPERIMENTAL PROCEDURES

Animals

A total of 47 adult female Sprague-Dawley rats (body wt 150–200 g, Anticimex, Stockholm) were used. Ten rats served as controls. Three rats were subjected to bilateral subdiaphragmatic truncal vagotomy and pyloroplasty^{13,16} and killed 3–4 weeks later. Three rats were given 6-hydroxydopamine (Hässel, Mölndal, Sweden) 100 mg/kg i.v. in the femoral vein 72 and 24 h before decapitation. Specimens from the stomach and from the small and large intestines were dissected out and processed for immunocytochemistry. Five rats were subjected to extrinsic denervation of 10–15 cm of the small intestine by clamping mesenteric blood vessels and surrounding mesentery for 10 min with a pair of forceps (nerve crushing).⁸ The animals were left for 10 days after the operation. Tissue specimens from the denervated intestinal loop were then processed for immunocytochemistry. Depletion of noradrenaline after clamping and chemical sympathectomy was verified by the Falck-Hillarp histofluorescence method.¹ Twenty-five rats were subjected to small intestinal myectomy, i.e. circumferential removal of 10 mm of the outer longitudinal smooth muscle layer together with adherent myenteric ganglia at the mid-jejunal level as described by Furness and Costa.⁹ These animals were killed 1 and 5 weeks after the operation. Segments were taken orally and anally to the lesion and processed for either immunocyto- or radioimmunochemistry.

Immunocytochemistry

The specimens from the stomach and from the small and large intestine were opened along the mesenteric border and pinned flat on a piece of balsa wood with the mucosal side facing the wood. They were fixed by floating in a mixture of 2% formaldehyde and 15% of a saturated aqueous picric acid solution in 0.1 M phosphate buffer (pH 7.2) overnight,

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Abbreviations: GRP, gastrin-releasing peptide.

and thoroughly rinsed in Tyrode buffer containing 10% sucrose. The outer (longitudinal) and inner (circular) muscle layers were separated and placed on chrome-alum-coated slides as whole mounts.⁴ The segments from myectomized rats were orientated in such a way that the oral and anal ends could be identified. Before further processing the whole mounts were dried, frozen and thawed in order to facilitate antibody penetration. Alternatively, the site of myectomy and the adjacent oral and anal segments were opened and cut serially in 5 mm pieces. These specimens and specimens from stomach, small and large intestines and from the externally denervated intestinal loop were immersed overnight in 4% formaldehyde in 0.1 M phosphate buffer, pH 7.2, and thoroughly rinsed in sucrose (10%, enriched Tyrode buffer). They were frozen on dry ice and transverse sections were cut in a cryostat at 10 μ m thickness. Sections and whole mounts were processed for the immunocytochemical demonstration² of galanin-like immunoreactivity using rabbit antisera raised against pure natural porcine galanin (code no. R2-8305) or synthetic porcine galanin (code no. 8416; the latter antiserum is commercially available from Milab, Malmö, Sweden). The antisera were used in dilution 1:200 (R2-8305) and 1:320 (8416). The site of the antigen-antibody reaction was revealed by application of fluorescein isothiocyanate-labelled pig anti-rabbit immunoglobulin G (Daco, Copenhagen) diluted 1:30. For controls we used antiserum which had been inactivated by the addition of excess amounts of antigen (10–100 μ g synthetic porcine galanin per ml diluted antiserum). The antisera were not inactivated by the 1–10 N-terminal fragment of galanin (10–100 μ g of peptide per ml diluted antiserum) suggesting that both antisera recognize sequences in the residual portion of galanin. However, antiserum R2-8305 recognizes both the N-terminal 1–20 fragment and the C-terminal 21–29 fragment (purified by high pressure liquid chromatography after tryptic digestion of pure natural porcine galanin) (Rökæus *et al.*, to be published). The antisera did not cross-react with other known or putative neuropeptides such as substance P, physalamin, vasoactive intestinal polypeptide, cholecystokinin, somatostatin, GRP, [Met]enkephalin neuropeptide Y or neurotensin (10–100 μ g added per ml diluted antiserum). Nevertheless, cross-reactivity with other peptides or proteins sharing immunodeterminants with galanin cannot be excluded. Therefore it is appropriate to refer to the immunoreactive material as galanin-like rather than galanin especially as the serial dilution curve for rat galanin does not parallel that for porcine galanin.¹⁴ For brevity, however, the shorter term is sometimes used in the following.

Radioimmunoassay

In four rats subjected to small intestinal myectomy segments of the intestine on both sides of the lesion were opened and cut serially in 5 mm specimens, 4 orally to the lesion, one at the lesion and 7 anally to the lesion. The 5-mm specimens were weighed, frozen on dry ice and kept at -20°C until extraction in boiling redistilled water. The specimens were minced, sonicated and extracted again in 1 M acetic acid at 4 $^{\circ}\text{C}$. Following centrifugation, lyophilization of the supernatant and addition of 0.9% saline containing 0.5% bovine serum albumin, the samples were analysed for galanin-like immunoreactivity with the antiserum R2-8305.¹⁴ The antiserum was used at a final dilution of 1:10,000 together with roughly 2000 cpm of ^{125}I -labelled galanin. The radioimmunoassay protocol has been described elsewhere.¹⁴ The mixture was incubated for 20–26 h at 4 $^{\circ}\text{C}$. Free and bound tracer were separated with plasma-coated charcoal. The assay is able to measure porcine galanin concentrations as low as 10–15 pmol in the tube. In this study pure natural bovine galanin was used as standard in the radioimmunoassay, paralleling more closely the dilution curve of the extracted rat galanin-like material than the previously¹⁴ used porcine galanin.

Materials

Synthetic porcine galanin was purchased from Peninsula, Belmont, California. Natural bovine galanin was isolated in the laboratory of Professor V. Mutt, Karolinska Institute (Carlquist and Rökæus, to be published). Porcine galanin 1–10 was synthesized by Dr J. Trojnar, Ferring Pharmaceuticals, Malmö, Sweden.

RESULTS

Immunocytochemical findings

As studied in control rats delicate varicose galanin-immunoreactive fibers were regularly observed in the smooth muscle and in the myenteric ganglia of the stomach and intestines (Figs 1–3). The longitudinal smooth muscle layer had comparatively few galanin-immunoreactive fibers, the myenteric ganglia had many and the circular smooth muscle layer had a fair number, with a tendency to forming a deep muscular plexus in the small intestine (Fig. 2C). The nerve fibers were generally orientated along the muscle

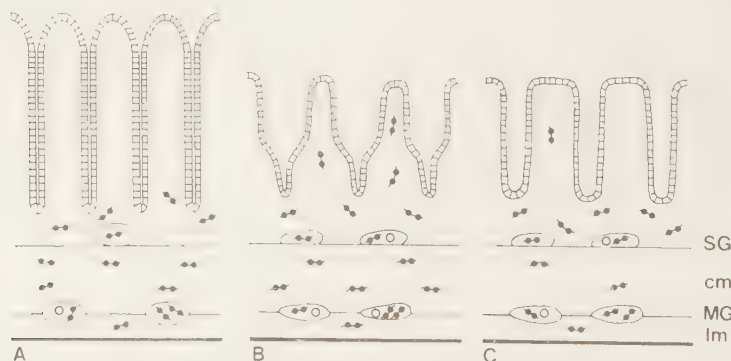


Fig. 1. Schematic outline of the topography and relative density of galanin-containing nerve fibers (●) and cell bodies (○) in the stomach (A), small intestine (B) and large intestine (C) of the rat. SG, submucosal ganglia; cm, circular muscle; MG, myenteric ganglia; lm, longitudinal muscle.

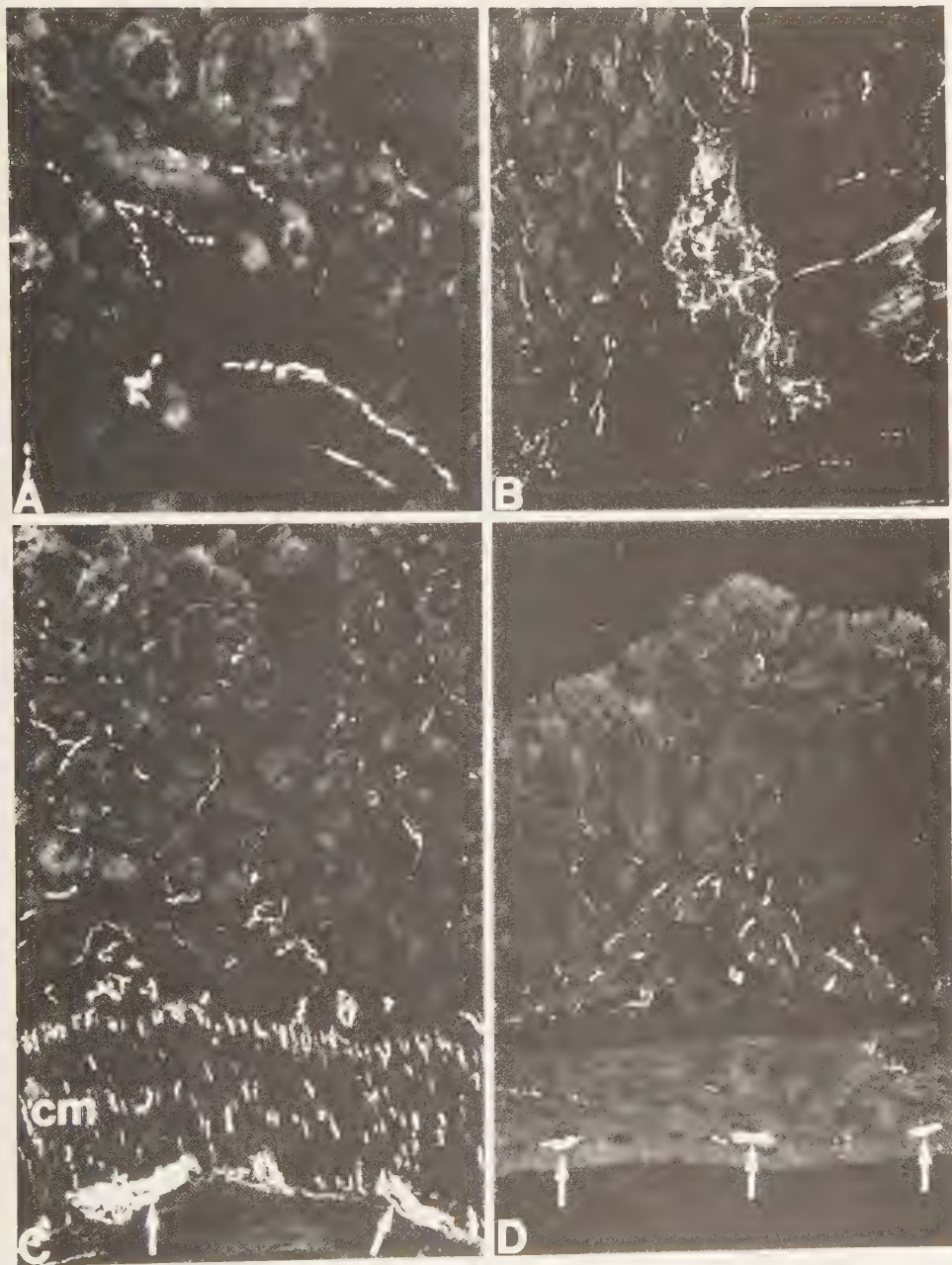


Fig. 2. Cryostat sections from rat digestive tract. (A) Oxyntic mucosa with galanin-immunoreactive nerve fibers in the submucosa and around the basal portion of the glands. (B) Myenteric ganglia with numerous galanin-immunoreactive fibers in the stomach. Scattered fibers in the smooth muscle. (C) Small intestine where galanin-immunoreactive fibers are particularly numerous in the myenteric ganglia (arrows) and in the circular smooth muscle layer (cm). A moderate number of fibers can be demonstrated in the submucosa and in the lamina propria. (D) In the large intestine galanin-immunoreactive fibers predominate in the myenteric ganglia (arrows) and in the submucosa (A, $\times 200$; B, $\times 175$; C and D, $\times 150$).

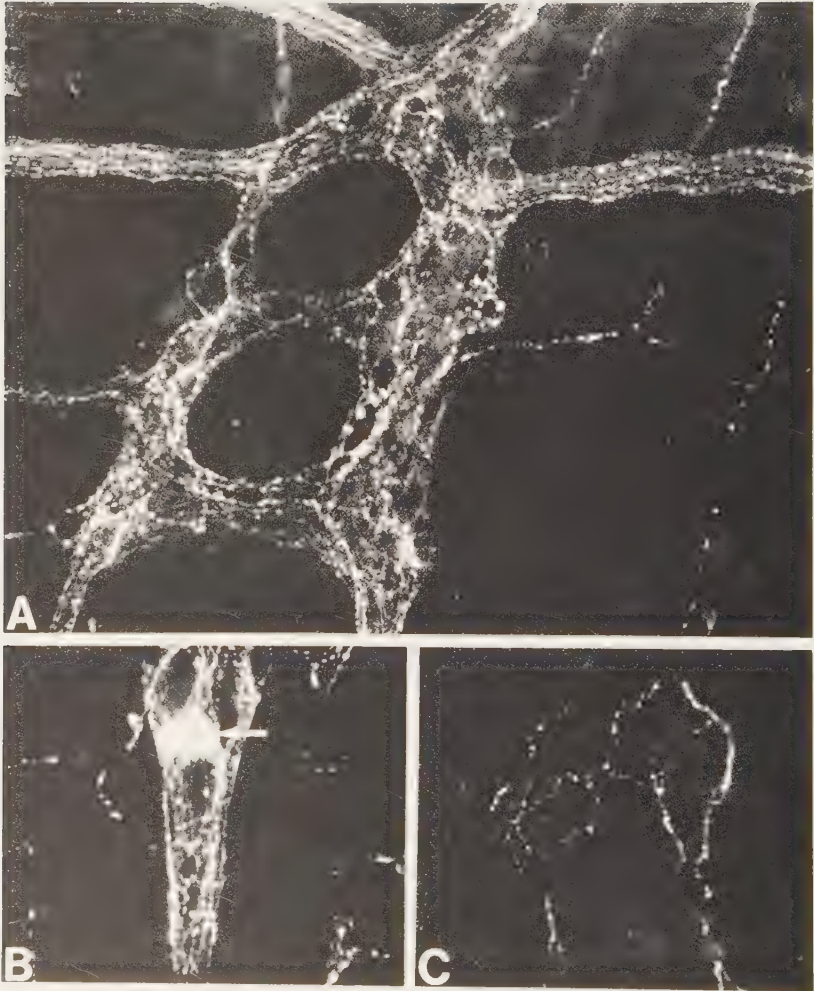
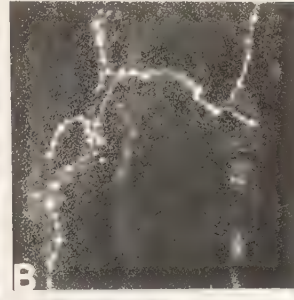
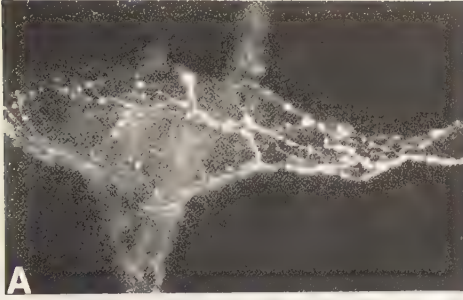


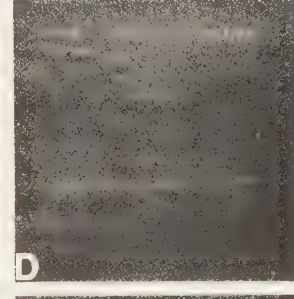
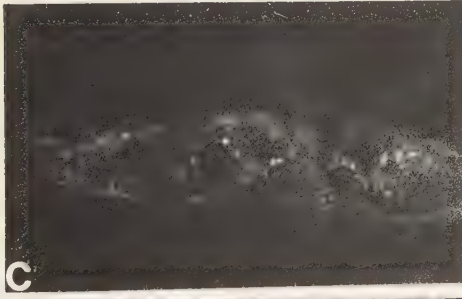
Fig. 3. Whole mounts of rat small intestine. (A and B) Longitudinal smooth muscle with adherent myenteric ganglia. A galanin-immunoreactive nerve cell body is shown in (B) (arrow). (C) Circular smooth muscle with a network of delicate galanin-immunoreactive fibers (A and C, $\times 200$; B, $\times 250$).

longitudinal smooth muscle

circular smooth muscle

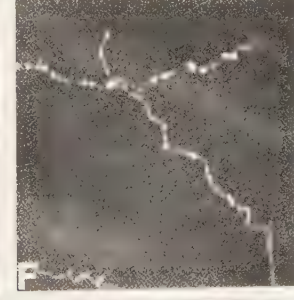
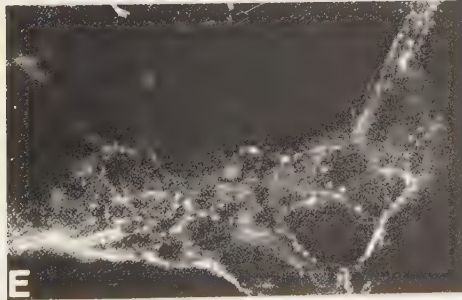


2 mm
orally

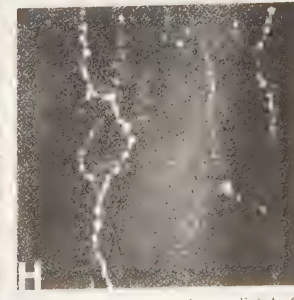
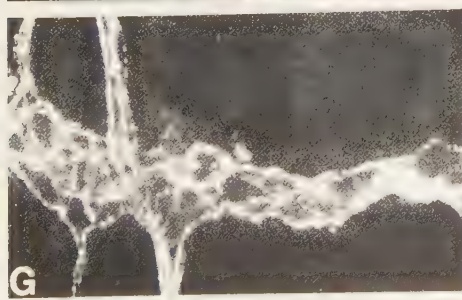


anally
↓

5 mm



10 mm



25 mm

Fig. 4. Rat jejunum. Whole mounts of longitudinal smooth muscle (with myenteric ganglia) A, C, E and G) and circular smooth muscle (B, D, F and H) one week after myectomy. (A and B) Two millimetres orally from the lesion. The galanin-immunoreactive fibers show normal number and distribution. Immediately distal to the lesion no galanin-immunoreactive fibers can be detected in the smooth muscle (not shown). (C and D) Five millimetres anally. Very few and weakly immunofluorescent galanin fibers can be detected predominantly in the myenteric ganglia. Further anally the galanin-immunoreactive fibers increase gradually in number. (E and F) Ten millimetres anally. Galanin-immunoreactive fibers occur in moderate number and are weakly immunofluorescent. At 15–20 mm anally the galanin-immunoreactive fibers have returned to normal distribution, frequency and immunostaining intensity (not shown) (G and H) Numerous intensely immunofluorescent galanin fibers 25 mm anally to the lesions (A–H, $\times 225$)

fibers although with frequent branching (Fig. 3C). Galanin-immunoreactive nerve fibers were few in the mucosa and submucosa of the stomach. In the small and large intestine galanin-immunoreactive fibers were quite numerous in the mucosa and the submucous ganglia. The regional and topographical distribution of the galanin fibers is illustrated in Fig. 1; there was a tendency to a gradual increase in the number of such fibers distally in the small intestine. The density of innervation was lower in the colon than in the small intestine; this was the case both in the smooth muscle and in the mucosa. Blood vessels in the gut wall received a moderate supply of galanin-immunoreactive nerve fibers. Moderately immunoreactive galanin nerve cell bodies were regularly seen in the myenteric ganglia throughout the gut (Fig. 3) and in the submucous ganglia of the small and large intestines. The two galanin antisera used gave identical results.

The distribution of galanin-immunoreactive fibers in the gut wall was virtually unaffected by vagotomy, chemical sympathectomy and extrinsic denervation (clamping) (not shown). After myectomy of the jejunum the galanin-immunoreactive fibers disappeared from the underlying circular smooth muscle layer but not from the mucosa. Immediately orally to the myectomy (within 2 mm) the number of galanin-immunoreactive fibers had decreased slightly in the myenteric ganglia. Further orally the number and distribution of galanin-immunoreactive fibers were normal (Fig. 4A). No overt loss of galanin-immunoreactive fibers could be detected in the circular smooth muscle at any point orally to the myectomy (Fig. 4B). Within an area of approximately 5 mm orally to the myectomy there was a lack of galanin-immunoreactive fibers in the myenteric ganglia and the smooth muscle. Beyond this point the galanin-immunoreactive nerve fibers returned gradually (Figs 4C–F). At 15–20 mm anally to the myectomy the number of galanin-immunoreactive fibers in the myenteric ganglia and smooth muscle layers was back to normal (Figs 4G–H). The loss of galanin-immunoreactive nerve fibers was equally striking 1 and 5 weeks after the operation. Myenteric and submucosal galanin-immunoreactive nerve cell bodies occurred in the same number and with the same immunofluorescence intensity above and below the lesion.

Radioimmunoassay findings

The galanin concentrations in the series of 5 mm segments taken orally and anally to the lesion are shown in Fig. 5. A marked reduction of the galanin concentration was noted in the gut wall underlying the myectomy as well as in the whole wall up to 15 mm anally. In addition the concentration of galanin was slightly reduced (not statistically significant) in the specimens taken immediately orally to the lesion.

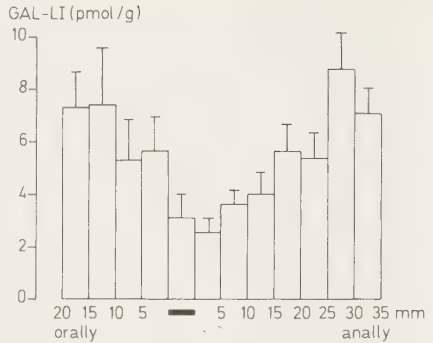


Fig. 5. Concentration of galanin-like immunoreactive (GAL-LI) material (expressed as pmol bovine galanin equivalents per g wet weight) in 5 mm segments of the rat jejunum taken orally and anally to the site of myectomy (indicated by horizontal line). Values are means $\pm$ SE ($n = 4$).

DISCUSSION

Galanin is a 29 amino acid peptide recently isolated from the porcine upper small intestine by virtue of its C-terminal amide structure.¹⁸ Galanin-like immunoreactivity has been demonstrated in both the central and peripheral nervous system of the rat.¹⁴ Immunocytochemistry revealed a dense network of galanin-immunoreactive nerve fibers in the submucosa, smooth muscle layers and intramural ganglia throughout the rat gastrointestinal tract. In the smooth muscle the density of innervation was lower in the colon than in the small intestine. In the mucosa galanin-immunoreactive fibers were quite numerous in the small intestine, less numerous in the large intestine and rare in the stomach. The galanin concentration and the frequency of galanin-immunoreactive nerve fibers gradually increased distally in the small intestine, as observed previously in several mammalian and submammalian species (Refs 12 and 14 and Ekblad *et al.*, to be published). In the present study of the rat gastrointestinal tract, galanin-immunoreactive nerve cell bodies could be demonstrated in both the myenteric and submucous ganglia. Various denervation procedures (surgical vagotomy, chemical sympathectomy and extrinsic denervation by clamping) failed to affect the distribution and frequency of enteric galanin-immunoreactive nerve fibers. These observations suggest that the vast majority of enteric galanin-immunoreactive nerve fibers are intrinsic to the gut wall. Local removal of myenteric ganglia (myectomy) at the mid-jejunal level caused loss of galanin-immunoreactive fibers anally to the lesion (for a distance of 15–20 mm) in both the myenteric ganglia and the smooth muscle, indicating long descending projections. There was no loss of galanin-immunoreactive fibers in the mucosa, hence mucosal fibers probably originate in the submucous ganglia. The denervation pattern of the smooth

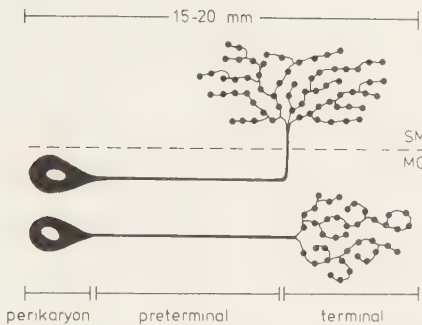


Fig. 6. Schematic illustration of the descending projections and the two sites of termination for myenteric galanin neurones. The preterminal portions of the neurones cannot be detected by the immunocytochemical procedure, probably because of a low galanin content. SM, smooth muscle; MG, myenteric ganglia.

muscle suggests that the majority of the descending axons run within the ganglionic layer for some distance before entering the smooth muscle. In fact there is good evidence for two sites of termination, one within the myenteric ganglia and another within the smooth muscle layers (Fig. 6). The projection pattern is reminiscent of that of the myenteric GRP neurones.^{5,6} The anal projections are long compared with those of several other peptide-containing neurones, e.g. those storing substance P, vasoactive intestinal polypeptide, somatostatin, enkephalin and neuropeptide Y, which issue processes in the 2–5 mm range.^{9,10} The only peptide-containing neurones known to have longer projections than the galanin neurones are those storing GRP; they project up to 20–25 mm in the anal direction.^{5,6} The distribution of

the galanin-immunoreactive fibers within the intestinal wall suggests an involvement of galanin in the control of epithelial functions and motility. In view of the long descending projections of the myenteric galanin neurones and their apparent dual termination in myenteric ganglia and in the smooth muscle, an integrative role in the control of motor activating circuits can be proposed. The results of recent *in vitro* studies on the effect of galanin on intestinal smooth muscle^{2,18} are compatible with such an action. In these studies galanin was shown to have multiple effects; a direct contractile effect on intestinal smooth muscle (rat small intestine) and a neuromodulatory effect, reflected in the suppression of the release of acetylcholine and substance P (guinea-pig taenia coli).⁷ Based on the anticipated suppressive effect of galanin⁷ and the excitatory effect of GRP⁵ on some myenteric neurones on one hand and on the similar projection pattern of the two neuronal systems on the other, it is tempting to speculate that they are complementary to each other in the regulation of motor neurone activity.

Conclusion

Enteric galanin-immunoreactive neurones represent a novel neuronal system with a possible role in the regulation of secretion and smooth muscle activity. A role in the control of motility is supported by the observation that myenteric galanin-immunoreactive neurones are numerous and project anally over a distance of 15–20 mm. Such a long projection distance suggests a coordinating involvement of galanin in complex motor integrative circuits.

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REFERENCES

1. Björklund A., Falck B. and Owman Ch. (1972) Fluorescence microscopic and microspectrofluorometric techniques for the cellular localization and characterization of biogenic amines. In *Methods of Investigative and Diagnostic Endocrinology* (ed. S. A. Berson), Vol. 1, pp. 318–368. North-Holland, Amsterdam.
2. Coons A. H., Leduc E. H. and Conolly J. M. (1955) Studies on antibody production. I. A method for the histochemical demonstration of specific antibody and its application to a study of the hyperimmune rabbit. *J. exp. Med.* **102**, 49–60.
3. Costa M. and Furness J. B. (1982) Neuronal peptides in the intestine. *Br. med. Bull.* **38**, 247–252.
4. Costa M., Buffa R., Furness J. B. and Solcia E. (1980) Immunohistochemical localization of polypeptides in peripheral autonomic nerves using whole mount preparations. *Histochemistry* **65**, 157–165.
5. Costa M., Furness J. B., Yanaihara N., Yanaihara C. and Moody T. W. (1984) Distribution and projections of neurones with immunoreactivity for both gastrin-releasing peptide and bombesin in the guinea-pig small intestine. *Cell Tissue Res.* **235**, 285–293.
6. Ekblad E., Ekman R., Håkanson R. and Sundler F. (1984) GRP neurones in the rat small intestine issue long anal projections. *Regul. Peptides* **9**, 279–288.
7. Ekblad E., Håkanson R., Sundler F. and Wahlestedt C. (1985) Galanin: neuromodulatory and direct contractile effects on smooth muscle preparations. *Br. J. Pharmac.* (in press).
8. Furness J. B. and Costa M. (1978) Distribution of intrinsic nerve cell bodies and axons which take up aromatic amines and their precursors in the small intestine of the guinea pig. *Cell Tissue Res.* **188**, 527–543.
9. Furness J. B., Costa M., Llewellyn-Smith I. J., Franco R. and Wilson A. J. (1981) Polarity and projections of peptide-containing neurones in the guinea-pig small intestine. In *Cellular Basis of Chemical Messengers in the Digestive System* (eds Grossman M., Brazier M. and Lechago J.), pp. 201–213. Academic Press, New York.
10. Furness J. B., Costa M., Emson P. S., Håkanson R., Moghizadeh E., Sundler F., Taylor I. L. and Chance R. E. (1983) Distribution, pathways and reaction to drug treatment of nerves with neuropeptide Y- and pancreatic polypeptide-like immunoreactivity in the guinea-pig digestive tract. *Cell Tissue Res.* **234**, 71–92.
11. Hökfelt T., Johansson O., Ljungdahl Å., Lundberg J. M. and Schultzberg M. (1980) Peptidergic neurones. *Nature* **284**, 515–521.

12. Melander T., Hökfelt T., Rökaeus A., Fahrenkrug J., Tatemoto K. and Mutt V. (1985) Distribution of galanin-like immunoreactivity in the gastrointestinal tract of several mammalian species. *Cell Tissue Res.* **239**, 253–270.
13. Nylander G. and Wikström S. (1967) Gastric emptying and propulsive intestinal motility following partial gastric resection, gastro-entero-anastomosis and abdominal trunk vagotomy in the rat. *Acta chir. scand.* **133**, 41–48.
14. Rökaeus A., Melander T., Hökfelt T., Lundberg J. M., Tatemoto K., Carlquist M. and Mutt V. (1984) A galanin-like peptide in the central nervous system and intestine of the rat. *Neurosci. Lett.* **47**, 161–166.
15. Schultzberg M., Hökfelt T., Nilsson G., Terenius L., Rehfeld J. F., Brown M., Elde R., Goldstein M. and Said S. (1980) Distribution of peptide- and catecholamine containing neurones in the gastro-intestinal tract of rat and guinea-pig: immunohistochemical studies with antisera to substance P, vasocative intestinal polypeptide, enkephalines, somatostatin, gastrin/cholecystokinin, neurotensin and dopamine β -hydroxylase. *Neuroscience* **5**, 689–744.
16. Shay H., Komarow S. A. and Gruenstein M. (1949) Effects of vagotomy in the rat. *Archs Surg. Chicago*, **59**, 210–213.
17. Sundler F., Håkanson R., Leander S. and Uddman R. (1982) Neuropeptides in the gut wall: cellular and subcellular localization, topographic distribution and possible physiological significance. In *Cytochemical Methods in Neuroanatomy* (eds Chan-Palay V. and Palay S. L.), pp. 341–356. Alan R. Liss, New York.
18. Tatemoto K., Rökaeus A., Jörnvall H., McDonald T. J. and Mutt V. (1983) Galanin—a novel biologically active peptide from porcine intestine. *FEBS Lett.* **164**, 124–128.

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PROJECTIONS OF PEPTIDE-CONTAINING NEURONS
IN RAT SMALL INTESTINE

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Abstract—The distribution, origin and projections of nerve fibers containing vasoactive intestinal peptide, neuropeptide Y, somatostatin, substance P, enkephalin and calcitonin gene-related peptide were studied in the rat jejunum by immunocytochemistry and immunochemistry. Their origin was determined by the use of various procedures for extrinsic denervation (chemical sympathectomy, bilateral vagotomy or clamping of mesenteric nerves). The terminations of the different types of intramural nerve fibers were identified by examination of the loss of nerve fibers that followed local disruption of enteric nervous pathways (intestinal myectomy, transection or clamping). The majority of the peptide-containing nerve fibers in the gut wall were intramural in origin, each nerve fiber population having its own characteristic distribution and projection pattern. Nerve fibers emanating from the myenteric ganglia terminated within the myenteric ganglia and in the smooth muscle layers: those storing vasoactive intestinal peptide/neuropeptide Y, somatostatin and substance P were descending, those storing enkephalin were ascending and those containing calcitonin gene-related peptide projected in both directions. Nerve fibers emanating from the submucosal ganglia terminated mainly within the submucosal ganglia and in the mucosa: those storing calcitonin gene-related peptide or vasoactive intestinal peptide/neuropeptide Y were ascending and those storing substance P or somatostatin were both ascending and descending. Enkephalin nerve fibers could not be detected in the mucosa.

The gastrointestinal tract harbors a wide variety of peptide-containing nerve fibers, the majority of which derive from intramural ganglia (for reviews see Refs 16 and 33). Much work has been done to identify their distribution and origin (see e.g. Refs 10-12, 17, 22, 25-27, 29, 31, 34 and 35), but comparatively fewer studies have dealt with their projection patterns.^{6,9,12,15,23} The analysis of the polarities and terminations of the different enteric peptide-containing nerve fiber populations have involved the use of procedures for the localized severing of intrinsic nervous pathways.^{8,15,23}

The present study examines the origin and terminations of nerve fibers storing vasoactive intestinal peptide (VIP), neuropeptide Y (NPY), somatostatin, substance P (SP), enkephalin and calcitonin gene-related peptide (CGRP) in the rat jejunum.

EXPERIMENTAL PROCEDURES

Animals

Ninety adult female Sprague Dawley rats (body wt 150-200 g, Alab, Stockholm, Sweden) were used.

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Abbreviations: BAM 12P, bovine adrenal medulla dodecapeptide; CGRP, calcitonin gene-related peptide; FITC, fluorescein isothiocyanate; GIP, glucose-dependent insulin-releasing peptide; HPLC, high performance liquid chromatography; NPY, neuropeptide Y; PHI, peptide with N-terminal, histidine and C-terminal isoleucine amide; PP, pancreatic polypeptide; PYY, peptide with both N-terminal and C-terminal tyrosine, RIA, radioimmunoassay; SP, substance P; TRITC, tetramethylrhodamine isothiocyanate, VIP, vasoactive intestinal peptide.

Denervation procedures

Severing of extrinsic nerves. Five rats were subjected to bilateral subdiaphragmatic truncal vagotomy and pyloroplasty¹⁰ and killed 3-4 weeks later. Five rats were given 6-hydroxydopamine (Hassle, Mölndal, Sweden) 100 mg/kg i.v. 72 and 24 h before decapitation (chemical sympathectomy). Five rats were subjected to extrinsic denervation of an 8-10 cm segment of the jejunum by clamping mesenteric blood vessels and surrounding mesentery for 10 min with a pair of forceps ('nerve crush').¹¹ The animals were left for 10 days after the operation.

Severing of intrinsic nervous pathways. Twenty rats were subjected to small intestinal myectomy, i.e. circumferential removal of 10 mm of the outer longitudinal smooth muscle layer together with adherent myenteric ganglia at the mid-jejunal level as described by Furness *et al.*¹⁵ Twenty rats were subjected to total disruption of enteric nerve fiber pathways by transection of the jejunum and subsequent re-joining of the severed parts by suturing end-to-end. Twenty rats were subjected to total disruption of enteric nerve fiber pathways by circumferential clamping of the jejunum (3-4 mm for 10 min) with a pair of forceps. This technique was adapted from Cowen *et al.*¹ who used it for the local denervation of blood vessels. All animals were killed 7-10 days after surgery.

The myectomy severed the nervous pathways connecting the myenteric ganglia leaving the submucosal ganglia unaffected. This procedure eliminates also the extrinsic innervation. It cannot be excluded that some of the myenteric neurons remained attached to the circular muscle after this operation. Such retained cell bodies could not be detected by immunocytochemistry, however. The transection and intestinal clamping severed the pathways in both myenteric and submucosal ganglia. The disadvantage of the transection is that the anastomoses result in scar formation over the sutures for ≥ 3 mm on either side of the lesion. As a consequence this technique is unsuitable for the study of neurons with short projections. Intestinal clamping, on the other hand, produced a well-defined demarcation line separating clamped (fibrotic) from non-clamped (non-fibrotic) tissue. This technique therefore is well suited for studies of short projections.

Table 1. Details of the antisera

Antigen	Code	Raised against	Working dilution		Source	Ref.
			Cryostat sections	Whole mounts		
VIP	7852	Unconjugated pure natural porcine VIP	1:640	1:320	Milab, Malmö, Sweden	9, 32
NPY*	8404	Unconjugated synthetic porcine NPY	1:320	1:160	Milab, Malmö, Sweden	—
	NPYY2	Protein-conjugated porcine NPY	1:400	1:200	P.C. Emson, MRC, Cambridge, U.K.	9, 34
Somatostatin	KB18	Protein-conjugated synthetic ovine somatostatin	1:800	1:400	Milab, Malmö, Sweden	11
[Met]enkephalin†	[Met]enk	Protein-conjugated synthetic [Met]enkephalin	1:320	1:160	R. J. Miller and K. J. Chang, Burroughs Wellcome Research Triangle Park, NJ, U.S.A.	1, 11
Substance P	SP8	Protein-conjugated synthetic bovine substance P	1:320	1:160	P. C. Emson, Cambridge, U.K.	2, 35
CGRP	8427	Protein-conjugated synthetic CGRP	1:640	1:320	Milab, Malmö, Sweden	35

*This antiserum does not cross-react with avian pancreatic polypeptide, bovine pancreatic polypeptide, peptide YY, VIP or PHI. The two NPY antisera produced identical results.

†The [Met]enkephalin antisera recognizes also [Leu]enkephalin.

Immunocytochemistry

Tissue specimens were collected from the jejunum of unoperated and operated rats. Segments were taken at the mid-jejunal level; in the animals with severed enteric nervous pathways segments were taken orally and anally to the lesion. The specimens were opened along the mesenteric border and pinned flat on a piece of balsa wood with the mucosal side facing the wood. The segments were orientated in such a way that the oral and anal ends could be identified. The specimens were fixed by floating in a mixture of 2% formaldehyde and 15% of a saturated aqueous picric acid solution in 0.1 M phosphate buffer (pH 7.2) overnight and thoroughly rinsed in Tyrode buffer containing 10% sucrose. The specimens were processed for immunocytochemistry either as whole mounts or as cryostat sections. The outer (longitudinal) and inner (circular) smooth muscle layers and the submucosa were separated and placed on chrome-alum-coated slides as whole mounts. Before further processing the whole mounts were dried, frozen and thawed to facilitate antibody penetration. Alternatively, specimens were frozen on dry ice and longitudinal sections were cut in a cryostat at 10 μ m thickness. Sections and whole mounts were processed for the immunocytochemical demonstration of VIP, NPY, SP, somatostatin, enkephalin and CGRP using previously characterized rabbit antisera (Table 1) and the indirect immunofluorescence method of Coons *et al.*³ It should be emphasized that immunocytochemical detection of peptides in neurons requires a minimum peptide concentration and that the actual concentration may vary locally. To reveal coexistence of VIP and NPY in the same nerve fibers, sections and whole mounts were first stained for NPY or VIP, examined in a fluorescence microscope and photographed. The antibodies were then eluted with acid potassium permanganate for 30–60 s.³⁷ The completeness of the elution was tested by application of fluoresceinated anti-rabbit immunoglobulin G. Sections devoid of immunofluorescence were then processed for the immunocytochemical demonstration of VIP or NPY and again photographed. To reveal coexistence of SP and CGRP sections and whole mounts were stained simultaneously with a guinea-pig antiserum against SP (code no JK1, kindly provided by Dr P. Emson, Cambridge, U.K.) and a rabbit

antiserum against CGRP (Table 1). The site of the antigen-antibody reaction was visualized in the case of SP by biotinylated antibodies to guinea-pig IgG raised in goat followed by exposure to fluorescein isothiocyanate (FITC)-conjugated avidin, and in the case of CGRP by tetramethylrhodamine isothiocyanate (TRITC)-conjugated antibodies to rabbit IgG raised in pig. Filters (Zeiss Nos 09 and 15) were shifted to enable selective excitation at 485 and 546 nm of the two fluorophores. For controls, antisera that had been inactivated by the addition of an excess amount of antigen (10–100 μ g of synthetic peptide/ml diluted antiserum) were used. Synthetic VIP, NPY, [Met]enkephalin, SP and CGRP were purchased from Peninsula, Belmont, CA, U.S.A. Synthetic somatostatin was given to us by Ferring Pharmaceuticals, Malmö, Sweden. Cross-reaction with other peptides or proteins containing amino acid sequences recognized by the different antisera cannot be excluded. It is appropriate, therefore, to refer to the immunoreactive material as VIP-like, NPY-like and so on. For simplicity, however, the shorter terms are used in the following.

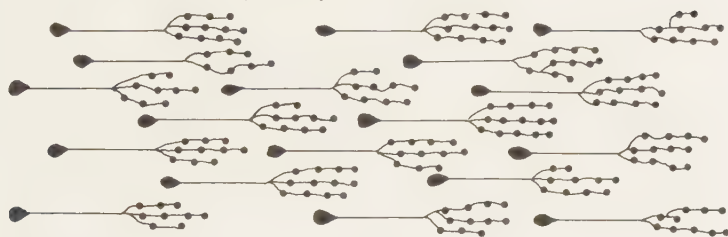
Estimation of nerve fiber length

The projection distance of each nerve fiber population was established after severing of intrinsic enteric pathways by measuring the distance from the lesion to the region showing a normal supply of nerve terminals. This distance comprised a region lacking nerve terminals (this region contains nerve cell bodies and preterminals with a peptide content at or below the detection limit of the technique) and a region showing a gradual return of nerve terminals (these nerve terminals derive from nerve cell bodies immediately adjacent to the lesion) (Fig. 1). Measurements were performed using a micrometer scale inserted in the visual field. Both cryostat sections and whole mount preparations (at least 6 of each) were used to establish the projection distance of each of the nerve fiber populations. To avoid tissue shrinkage due to fixation, all specimens were flat, with a minimum of stretching, on a piece of balsa wood prior to fixation.

Radioimmunochemistry

The concentrations of CGRP, [Met]enkephalin, and NPY in extracts of intestinal specimens were determined by

Intact enteric nervous pathways



Disruption of nervous pathways

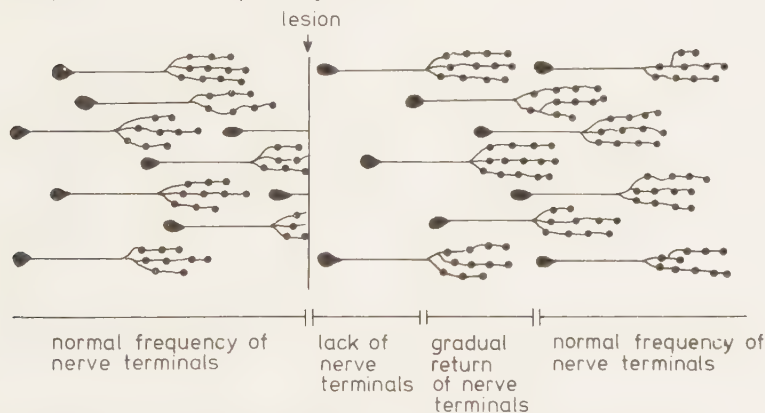


Fig. 1. Diagram illustrating the uni-directional projections of a hypothetical population of enteric neurons (a) and the proposed consequences of local disruption of the pathways (b). In the diagram it is assumed (perhaps erroneously) that all projections of this hypothetical neuronal population are of the same length. Cell bodies and preterminals have a low neuropeptide content, the nerve terminals are rich in the neuropeptide.

radioimmunoassay (RIA). The jejunal segments were opened, rinsed and cut serially in 5 mm specimens, 4 orally and 4 anally to the lesion. The specimens were weighed and boiled in redistilled water (0.1 g wet wt/ml) for 2 min. Following the addition of 3 M acetic acid to a final concentration of 0.5 M, the tissue was homogenized (Polytron, 1–2 min), and extracted at room temperature for 30 min. After centrifugation at 2000 g for 15 min the supernatants were collected and lyophilized. The material was freeze-dried and taken up in 2 ml of 0.05 M phosphate buffer (pH 7.4).

For RIA of CGRP we used a rabbit antiserum (code No. A13, Milab, Malmö, Sweden) raised against synthetic rat CGRP conjugated to bovine serum albumin by the carbodiimide reaction. The antiserum was used in a final dilution of 1:37,500. Tracer was (Tyr⁶)-rCGRP iodinated by the chloramine-T oxidation procedure. [¹²⁵I]Tyr-CGRP was purified by high performance liquid chromatography (HPLC). CGRP-immunoreactive material was extracted and determined by a RIA procedure with a detection limit of 20 pmol/l.²¹ There was no cross-reaction with human calcitonin, calcitonin or calcitonin C-terminal adjacent peptide. The interassay variation was below 11% in the

range 100–300 pmol/l. In the [Met]enkephalin-RIA we used a rabbit antiserum (R-26, a kind gift from Prof. K. Voigt, Freie Universität, Berlin) in a final dilution of 1:20,000.³⁸ The antibody is C-terminally directed. [¹²⁵I][Met]enkephalin was purified by HPLC; 10 pmol/l was the detection limit. The interassay variation was below 10% in the range 50–250 pmol/l. The antibody does not cross-react with [Leu]enkephalin, β -endorphin (1–31), β -endorphin (1–17), β -endorphin (1–9), [Met]enkephalin-Arg⁸-Phe⁷, dynorphin (1–8), BAM 12P (bovine adrenal medulla dodecapeptide) or Peptide E.³⁸ Immunoreactive NPY was determined using a rabbit antiserum raised against pure porcine NPY conjugated to bovine serum albumin (a kind gift from Dr P. C. Emson, Cambridge U.K.). It cross-reacts with PYY (peptide with both N-terminal and C-terminal tyrosine) to 100% but does not cross-react with PP (pancreatic polypeptide), GIP (glucose-dependent insulin-releasing peptide), PHI (peptide with N-terminal histidine and C-terminal isoleucine amide), VIP or secretin. The detection limit was 25 pmol/l and the interassay variation in the range 50–100 pmol/l was below 12%.¹³ Each tissue extract was assayed in serial dilutions in the respective neuropeptide assay.

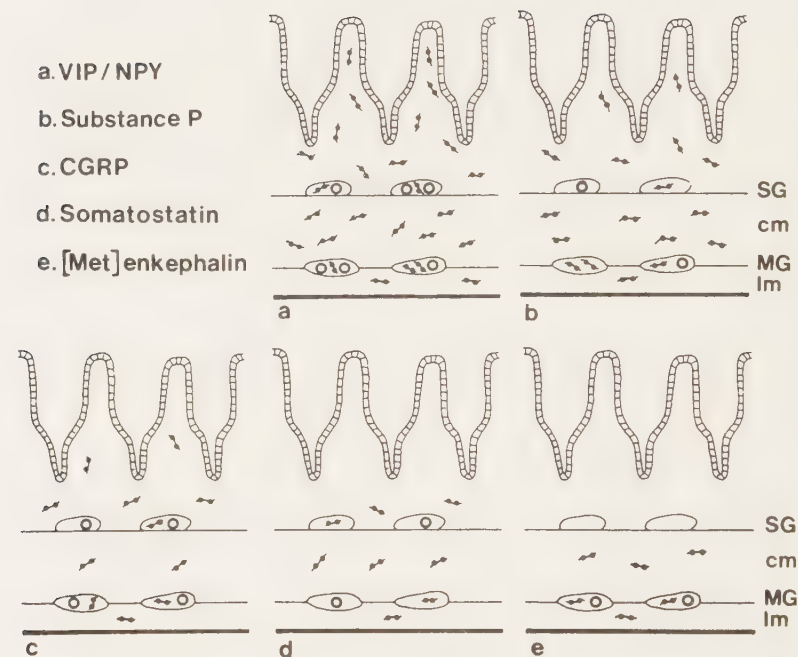


Fig. 2. Schematic outline of the topography and relative density of peptide-containing nerve fibers (●) and nerve cell bodies (○) in the rat jejunum. SG, submucosa; cm, circular muscle; MG, myenteric ganglia; lm, longitudinal muscle.

RESULTS AND COMMENTS

All the antisera used demonstrated neuronal elements in the wall of the rat jejunum. The results on the topography and distribution are summarized in Figs 2 and 3. In agreement with previous findings^{9,11} VIP and NPY were found to be co-localized in a prominent population of intramural neurons (Fig. 4). In addition, NPY (but not VIP) occurred in a population of fibers of extrinsic (sympathetic) origin, containing also noradrenaline (see also Refs 9, 11, 17 and 34). SP and CGRP occurred in separate and fairly prominent enteric nerve fiber populations. In addition, SP and CGRP were co-localized in a minor population of mainly perivascular nerve fibers of extrinsic (presumably sensory) origin (see also Ref. 35).

Extrinsic denervation

Vagotomy. Vagal denervation did not change either the density or the distribution of the various peptide-containing nerve fiber populations.

Sympathectomy. The distribution of peptide-

containing nerve fibers in the gut wall was virtually unaffected by sympathectomy with the exception of perivascular NPY fibers which disappeared.

Mesenteric clamping. This denervation procedure caused a loss of perivascular nerve fibers containing NPY or SP/CGRP. Other types of peptide-containing nerve fibers were unaffected.

Disruption of enteric nervous pathways. Distribution and projections of individual enteric neuronal systems

The various neuronal systems are described in order of over-all nerve fiber density.

Vasoactive intestinal peptide/neuropeptide Y neurons. Numerous intensely VIP- and NPY-immunoreactive, fine varicose nerve fibers could be demonstrated throughout the wall including the ganglia (Figs 2a and 3a, b). Nerve cell bodies, displaying a varying degree of immunoreactivity, were frequently seen in both the myenteric and submucosal ganglia (Figs 3a, b and 4). After myectomy or local disruption of nerve fiber pathways by intestinal clamping, VIP/NPY fibers were markedly reduced in number in myenteric ganglia and smooth muscle up

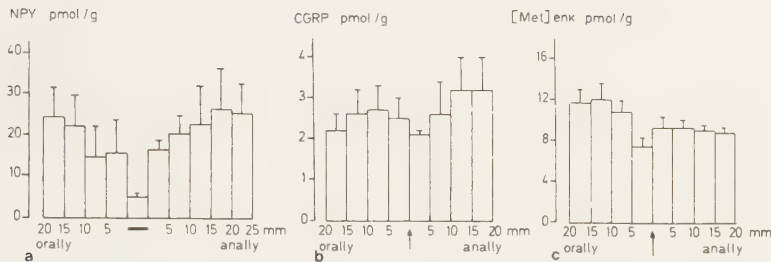


Fig. 7. Concentration of (a) NPY-like, (b) CGRP-like and (c) [Met]enkephalin-like immunoreactive material (expressed as pmol equivalents of the standard peptide per g wet weight) in 5 mm segments of the rat jejunum taken orally and anally to the lesion: (a) myectomy (indicated by horizontal line) and (b and c) intestinal clamping (indicated by arrow). Values are means $\pm$ SE; $n = 6$ (a), $n = 13$ (b), $n = 4$ (c).

to 2 mm anally to the lesion (Fig. 5). Further distally the nerve fibers had a normal frequency. Orally to the lesion there was no loss of VIP/NPY nerve fibers in myenteric ganglia or smooth muscle. In the area underlying the myectomy there was a marked reduction in the number of VIP/NPY nerve fibers in the circular smooth muscle; the innervation pattern in the mucosa and submucosa was normal (Fig. 6). The concentration of NPY was markedly reduced in the area underlying the myectomy. No statistically significant reduction of the NPY concentration could be detected in the oral or anal segments (Fig. 7a). Intestinal clamping caused a loss of VIP/NPY nerve fibers in the mucosa and submucosa up to 1–2 mm orally to the lesion with a gradual return further orally (Fig. 8). The nerve fiber density was back to normal at approximately 4 mm. No loss of VIP/NPY fibers could be demonstrated in the mucosa or submucosa anally to the lesion. Thus VIP/NPY neurons in the myenteric ganglia project anally (up to 2 mm) to innervate smooth muscle and myenteric ganglia. Submucous VIP/NPY immunoreactive neurons project orally (up to 4 mm), terminating in the mucosa, submucosa and submucous ganglia. They probably send projections also to the circular muscle layer (since some VIP/NPY fibers could be detected in the area underlying the myectomy).

Substance P neurons. Numerous varicose SP-immunoreactive nerve fibers could be demonstrated in the smooth muscle layers and myenteric ganglia (Figs 2b and 9). A moderate number of SP fibers were found in the mucosa, submucosa and submucous ganglia (Figs 2b and 10). A low number of SP-immunoreactive nerve cell bodies could be detected in the intramural ganglia (Fig. 10). Myectomy or local nerve pathway disruption by transection or intestinal clamping resulted in a total loss of SP fibers in myenteric ganglia and smooth muscle up to 4 mm anally to the lesion (Fig. 9). Beyond this point there was a gradual return of SP fibers; 3 mm further anally the innervation was back to normal. Orally to the

lesion there was no loss of myenteric SP nerve fibers. In the area underlying the myectomy the SP nerve fibers had disappeared from the smooth muscle layer, but they were unchanged in number and distribution in the mucosa and submucosa. After total nerve fiber pathway disruption (intestinal clamping or transection) the number of SP fibers in the mucosa and submucosa was greatly reduced for approximately 4 mm in both oral and anal direction (Fig. 10). Thus, myenteric SP neurons issue descending projections (6–7 mm) to smooth muscle and myenteric ganglia. Submucous SP neurons project both orally and anally for a distance of 3–4 mm into the mucosa and submucosa.

Calcitonin gene-related peptide neurons. CGRP fibers were few in the smooth muscle layer. A moderate number of CGRP-immunoreactive nerve fibers were observed in the intramural ganglia as well as in the mucosa and submucosa (Figs 2c and 3c). CGRP-immunoreactive nerve cell bodies were moderate in number in the myenteric and submucous ganglia (Fig. 3c). Myectomy or intestinal clamping reduced the number of CGRP fibers in the myenteric ganglia and smooth muscle layers for a distance of 1–2 mm both orally and anally to the lesion (Fig. 11). Further orally or anally, the density of CGRP fibers was normal. There was no loss of nerve fibers in the mucosa or submucosa in the area underlying the myectomy. A few CGRP-immunoreactive nerve fibers could be detected in the circular muscle layer underlying the area of myectomy. After nerve fiber pathway disruption (by transection or intestinal clamping), CGRP fibers disappeared orally for approx. 4 mm in the mucosa and submucosa and for approx. 2 mm in the submucous ganglia. Beyond these points the density of CGRP fibers increased gradually and was back to normal 3–4 mm orally to the lesion in the submucous ganglia and 7–8 mm orally in the mucosa and submucosa (Fig. 12). There was no loss of CGRP in the mucosa and submucosa anally to the lesion. Chemical determinations failed

to reveal a reduction of the CGRP concentration in either oral or anal segments (Fig. 7b). Thus, myenteric CGRP neurons issue ascending and descending projections (1–2 mm) to myenteric ganglia and smooth muscle. Submucosal CGRP neurons project orally for 3–4 mm to other submucosal ganglia and for 7–8 mm to the mucosa and submucosa and probably also to some extent to the circular smooth muscle.

Somatostatin neurons. A moderate number of somatostatin-immunoreactive nerve fibers could be demonstrated in the intramural ganglia and in the submucosa and basal portion of the mucosa while only a few fibers occurred in the smooth muscle (Figs 2d and 3d). Occasionally, somatostatin-immunoreactive nerve cell bodies could be demonstrated in the intramural ganglia. After myectomy or intestinal clamping, the somatostatin fibers disappeared anally from the myenteric ganglia and smooth muscle for a distance of 2–3 mm (Fig. 13). A gradual return of somatostatin fibers could be observed from 3 to 6 mm anally; beyond this point they had a normal frequency. Somatostatin nerve fibers were lacking in the circular smooth muscle underlying the site of myectomy, whereas the mucosal and submucosal somatostatin nerve supply was unchanged. After intestinal clamping, somatostatin nerve fibers in the mucosa and submucosa disappeared for approx. 3 mm anally and orally to the lesion (Fig. 14). Beyond these points there was a gradual increase in the nerve fiber density over a distance of 1–2 mm. A normal frequency of mucosal and submucosal somatostatin nerve fibers was found 4–5 mm anally and orally to the lesion. Thus, myenteric somatostatin neurons issue descending projections 5–6 mm in length to myenteric ganglia and smooth muscle. The mucosa receives somatostatin fibers from the submucosa ganglia. These fibers project 4–5 mm in both oral and anal direction.

Enkephalin neurons. Enkephalin-immunoreactive nerve fibers could be demonstrated in moderate number in the myenteric ganglia and in low numbers in the smooth muscle layers (Figs 2e and 15). No such fibers could be detected in the mucosa, submucosa or submucosal ganglia. A moderate number of immunoreactive nerve cell bodies could be visualized in the myenteric ganglia, none were seen in the submucosal ganglia. Myectomy or intestinal clamping caused a total disappearance of enkephalin fibers in myenteric ganglia and smooth muscle up to 3 mm orally to the lesion (Fig. 15). Further orally, immunoreactive fibers gradually reappeared; the density of enkephalin fibers was back to normal 6–7 mm from the lesion. There was no change in the fiber density anally to the lesion. The [Met]enkephalin concentration measured by RIA was reduced in the first 5 mm segment orally to the site of intestinal clamping (Fig. 7c). Thus, enkephalin nerves originate in the myenteric ganglia and project for 6–7 mm in oral direction to innervate myenteric ganglia and smooth muscle.

DISCUSSION

The results of the present study together with those of previous reports on enteric neurons in the rat and guinea pig indicate that each nerve fiber population has its own characteristic distribution and projection pattern.^{4,6,8,12,15,17,18} In the rat, myenteric neurons seemed to project preferentially in the anal direction terminating in myenteric ganglia and smooth muscle; submucosal neurons on the other hand project in both directions, albeit preferentially orally, terminating in submucosal ganglia and in the mucosa.

When dealing with peptide-containing neuronal populations it has to be born in mind that two or more peptides may coexist in the same neuron. In the case of SP and neurokinin A and of VIP and PHI this is to be expected since they derive from the same precursors, preprotachykinin and prepro-VIP, respectively.^{35,36} More unexpected has been the observation of coexistence of neuropeptides expressed by entirely different genes. Such coexistence has been demonstrated for VIP and NPY,^{9,11} VIP and galanin,²⁸ SP and CGRP,^{20,35} and for the constellation NPY, CGRP, cholecystokinin and somatostatin.¹⁹ Apart from VIP and NPY, the peptides investigated in the present study, do not seem to coexist with each other in enteric neurons in the rat jejunum. Possible coexistence of the following pairs of neuropeptides in rat small intestine has been tested using double immunostaining techniques: SP/NPY, SP/VIP, SP/galanin, SP/enkephalin, GRP/NPY, GRP/VIP, GRP/galanin, GRP/enkephalin, CGRP/NPY, CGRP/VIP, CGRP/galanin, CGRP/GRP and CGRP/enkephalin. In no instance could coexistence of the two peptides in the same neuron be demonstrated (Ekblad *et al.*, unpublished observations). The various neuronal populations are therefore described as separate entities. However, the possibility that the peptides dealt with here coexist with each other in minority subpopulations of the various enteric neurons cannot be ruled out. Another complication when studying peptide coexistence in enteric nerve fibers is the tendency of many of these fibers to run close together in bundles (companion fibers) (Ref. 31; Ekblad *et al.*, unpublished observations).

In the present study ascending myenteric pathways were revealed for the enkephalin and CGRP neurons only. The finding of descending projections in myenteric ganglia of rat jejunum is consistent with previous observations in the guinea pig small intestine concerning neurons storing VIP,¹⁵ NPY,¹⁷ SP⁴ or somatostatin.¹⁵ However, myenteric SP neurons were found to give off ascending projections in the guinea pig,⁴ an observation which we failed to confirm in the rat. Myenteric enkephalin neurons have previously been found to issue both ascending and descending projections in the guinea pig small intestine.^{18,23} Interestingly, unlike all other known myenteric neuronal systems the enkephalin neurons had no descending projections in the rat. Based on the present and

previous results²² we conclude that the bulk of mucosal nerve fibers originate in submucous ganglia. In the guinea pig Keast *et al.*²² found submucous SP, NPY, somatostatin and cholecystokinin-containing neurons to issue short mucosal projections. The present results in the rat indicate that submucous VIP/NPY, SP, and somatostatin neurons give off short ascending projections; short descending SP and somatostatin projections could be seen as well. With respect to submucous CGRP neurons the prominent ascending (6–8 mm) mucosal projections are especially noteworthy.

CONCLUSION

The enteric neurons examined in the present study all seem to issue projections shorter than 8 mm. Two previously studied neuronal populations, namely

those storing gastrin releasing peptide (GRP) and galanin have been found to issue descending projections approx. 20 mm in length, terminating in myenteric ganglia and smooth muscle^{6,8,12} (Fig. 16).

The peristaltic wave has two components: a descending inhibition of smooth muscle activity generating relaxation and an ascending excitation giving rise to contractions (cf. Refs 5 and 24). The neuronal reflexes behind the peristaltic process can be understood only on the basis of a detailed knowledge of the neuronal circuits. Mapping out the projection and termination patterns of the various enteric neuronal populations is necessary before we can assess their significance in intestinal physiology.

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REFERENCES

- Alumets J., Häkanson R., Sundler F. and Chang K.-J. (1978) Leu-enkephalin-like material in nerves and enterochromaffin cells in the gut. *Histochemistry* **56**, 187–196.
- Brodin E., Alumets J., Häkanson R., Leander S. and Sundler F. (1981) Immunoreactive substance P in the chicken gut: distribution, development and possible functional significance. *Cell Tiss. Res.* **216**, 455–469.
- Coons A. H., Leduc E. H. and Connolly J. M. (1955) Studies on antibody production—I. A method for the histochemical demonstration of specific antibody and its application to a study of the hyperimmune rabbit. *J. exp. Med.* **102**, 49–60.
- Costa M., Furness J. B., Llewellyn-Smith I. J. and Cuello A. C. (1981) Projections of substance P-containing neurons within the guinea-pig small intestine. *Neuroscience* **6**, 411–424.
- Costa M. and Furness J. B. (1982) Nervous control of intestinal motility. In *Handbook of Experimental Pharmacology*, Vol. 59 (ed. Bertaccini G.), pp. 279–382. Springer-Verlag, Berlin.
- Costa M., Furness J. B., Yanaihara N., Yanaihara C. and Moody T. W. (1984) Distribution and projections of neurons with immunoreactivity for both gastrin-releasing peptide and bombesin in the guinea-pig small intestine. *Cell Tiss. Res.* **235**, 285–293.
- Cowen T., McCormick D. E. M., Toff W. D., Burnstock G. and Lumley J. S. P. (1982) The effect of surgical procedures on blood vessel innervation. *Blood Vessels* **19**, 65–70.
- Ekblad E., Ekman R., Häkanson R. and Sundler F. (1983) GRP neurones in the rat small intestine issue long anal projections. *Regul. Pept.* **9**, 279–287.
- Ekblad E., Häkanson R. and Sundler F. (1984) VIP and PHI coexist with an NPY-like peptide in intramural neurones of the small intestine. *Regul. Pept.* **10**, 47–55.
- Ekblad E., Wahlestedt C., Ekelund M., Häkanson R. and Sundler F. (1984) Neuropeptide Y in the gut and pancreas: distribution and possible vasomotor function. *Front. Horm. Res.* **12**, 85–90.
- Ekblad E., Ekelund M., Graffner H., Häkanson R. and Sundler F. (1985) Peptide-containing nerve fibres in the small stomach wall of rat and mouse *Gastroenterology* **89**, 73–85.
- Ekblad E., Rökæus Å., Häkanson R. and Sundler F. (1985) Galanin nerve fibers in the rat gut: origin and projections. *Neuroscience* **16**, 355–363.
- Ekman R., Widerlöv E., Wallius H. and Lindström L. H. (1984) Elevated levels of NPY-like material in CSF in depression. ISPN-XVth International Congress, Vienna, July 15–19, p. 73.
- Furness J. B. and Costa M. (1978) Distribution of intrinsic nerve cell bodies and axons which take up aromatic amines and their precursors in the small intestine of the guinea pig. *Cell Tiss. Res.* **188**, 527–543.
- Furness J. B., Costa M., Llewellyn-Smith I. J., Franco R. and Wilson A. J. (1981) Polarity and projections of peptide-containing neurons in the guinea pig small intestine. In *Cellular Basis of Chemical Messengers in the Digestive System* (eds Grossman M. I., Brazier M. A. B. and Lechago J.), pp. 201–213. Academic Press, New York.
- Furness J. B. and Costa M. (1982) Identification of gastrointestinal neurotransmitters. In *Handbook of Experimental Pharmacology*, Vol. 59 (ed. Bertaccini G.), pp. 383–460. Springer-Verlag, Berlin.
- Furness J. B., Costa M., Emson P. C., Häkanson R., Moghimiadeh E., Sundler F., Taylor I. L. and Chance R. E. (1983) Distribution, pathways and reactions to drug treatment of nerves with neuropeptide Y- and pancreatic polypeptide-like immunoreactivity in the guinea-pig digestive tract. *Cell Tiss. Res.* **234**, 71–92.
- Furness J. B., Costa M., and Miller R. J. (1983) Distribution and projections of nerves with enkephalin-like immunoreactivity in the guinea-pig small intestine. *Neuroscience* **8**, 653–664.
- Furness J. B., Costa M., Gibbins I. L., Llewellyn-Smith I. J. and Oliver J. R. (1985) Neurochemically similar myenteric and submucous neurons directly traced to the mucosa of the small intestine. *Cell Tiss. Res.* **241**, 155–163.
- Gibbins I. L., Furness J. B., Costa M., MacIntyre I., Hillyard C. J. and Gargis S. (1985) Co-localization of calcitonin gene-related peptide-like immunoreactivity with substance P in cutaneous, vascular and visceral sensory neurons of guinea pigs. *Neurosci. Lett.* **57**, 125–130.
- Grunditz T., Ekman R., Häkanson R., Rerup C., Sundler F. and Uddman R. (1986) Calcitonin gene-related peptide in the thyroid gland: Distribution and effects on thyroid hormone secretion. *Endocrinology*, in press.

22. Keast J. R., Furness J. B. and Costa M. (1984) Origins of peptide and norepinephrine nerves in the mucosa of the guinea pig small intestine. *Gastroenterology* **86**, 637–644.
23. Kobayashi S. and Nishisaka T. (1985) Myenteric enkephalin neurons around the Laser-photo-coagulation necrosis: an immunocytochemical investigation in the guinea pig jejunum and proximal colon. *Archs histol. jap.* **48**, 239–254.
24. Kosterlitz W. H. and Watt A. J. (1975) The peristaltic reflex. In *Methods in Pharmacology*, Vol. 3 (eds Daniel E. E. and Paton D. M.), pp. 391–401. Plenum, New York.
25. Leander S., Håkanson R. and Sundler F. (1981) Nerves containing substance P, vasoactive intestinal peptide, enkephalin or somatostatin in the guinea pig *taenia coli*. *Cell Tiss. Res.* **215**, 21–39.
26. Leander S., Ekman R., Uddman R., Sundler F. and Håkanson R. (1984) Neuronal cholecystokinin, gastrin-releasing peptide, neurotensin and β -endorphin in guinea-pig intestine. Distribution and possible motor function. *Cell Tiss. Res.* **235**, 521–531.
27. Malfors G., Leander S., Brodin E., Håkanson R., Holmin T. and Sundler F. (1981) Peptide containing neurones intrinsic to the gut wall. An experimental study in the pig. *Cell Tiss. Res.* **214**, 225–238.
28. Melander T., Hökfelt T., Rökaeus Å., Fahrenkrug J., Tatemoto K. and Mutt V. (1985) Distribution of galanin-like immunoreactivity in the gastrointestinal tract of several mammalian species. *Cell Tiss. Res.* **239**, 253–270.
29. Moghizadeh E., Ekman R., Håkanson R., Yanaihara N. and Sundler F. (1983) Neuronal gastrin-releasing peptide in the mammalian gut and pancreas. *Neuroscience* **10**, 553–563.
30. Nylander G. and Wikström S. (1967) Gastric emptying and propulsive in testinal motility following partial gastric resection, gastro-enteroanastomosis and abdominal trunk vagotomy in the rat. *Acta Chir. Scand.* **133**, 41–48.
31. Schultzberg M., Hökfelt T., Nilsson G., Terenius L., Rehfeld J. F., Brown M., Elde R., Goldstein M. and Said S. (1980) Distribution of peptide- and catecholamine-containing neurones in the gastro-intestinal tract of rat and guinea-pig: immunohistochemical studies with antisera to substance P, vasoactive intestinal polypeptide, enkephalins, somatostatin, gastrin/cholecystokinin, neurotensin and dopamine β -hydroxylase. *Neuroscience* **5**, 689–744.
32. Sjölund K., Sandén G., Håkanson R. and Sundler F. (1983) Endocrine cells in human intestine: an immunocytochemical study. *Gastroenterology* **85**, 1120–1130.
33. Sundler F., Håkanson R. and Leander S. (1980) Peptidergic nervous systems in the gut. *Clin. Gastroenterol.* **9**, 517–543.
34. Sundler F., Moghizadeh E., Håkanson R., Ekelund M. and Emson P. (1983) Nerve fibers in the gut and pancreas of the rat displaying neuropeptide-Y immunoreactivity. Intrinsic and extrinsic origin. *Cell Tiss. Res.* **230**, 487–493.
35. Sundler F., Brodin E., Ekblad E., Håkanson R. and Uddman R. (1985) Sensory nerve fibers: Distribution of substance P, neurokinin A and calcitonin gene-related peptide. In *Tachykinin Antagonists. Proc. Fernström Symp.*, Vol. 6 (eds Håkanson R. and Sundler F.), pp. 3–14. Elsevier, Amsterdam.
36. Sundler F., Ekblad E., Böttcher G., Alumets J. and Håkanson R. (1985) Coexistence of peptides in the neuroendocrine system. In *Biogenetics of Neurohormonal Peptides* (eds Håkanson R. and Thorell J.) pp. 213–243. Academic Press, London.
37. Tramu G., Pillez A. and Leonardelli J. (1980) An efficient method of antibody elution for the successive or simultaneous location of two antigens by immunocytochemistry. *J. Histochem. Cytochem.* **26**, 322–324.
38. Voigt K. H., Weber E. and Martin R. (1980) Neuropeptides, subcellular localization of ACTH and related peptides. In *Structure and Activity of Natural Peptides* (eds Voelter W. and Weitzel ?), pp. 115–139. Debrugter, Berlin.

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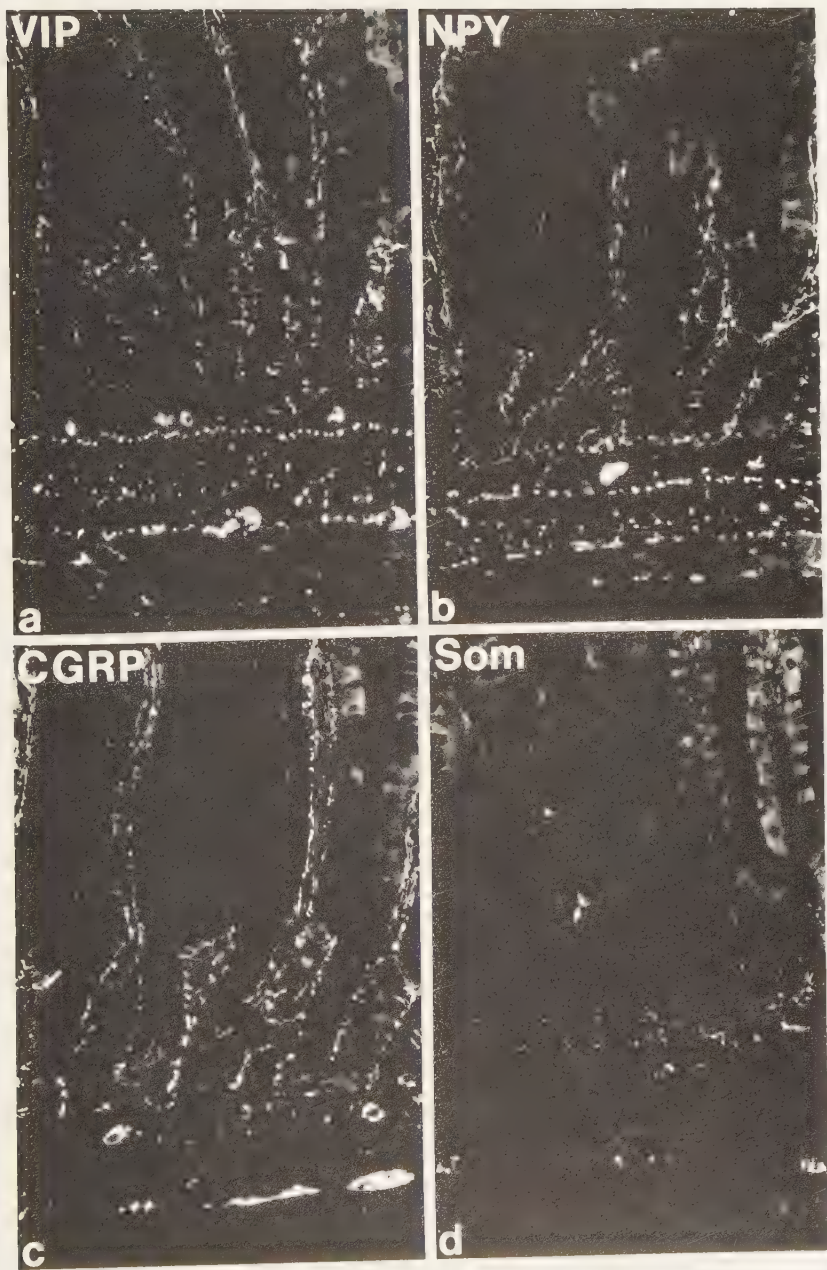


Fig.3

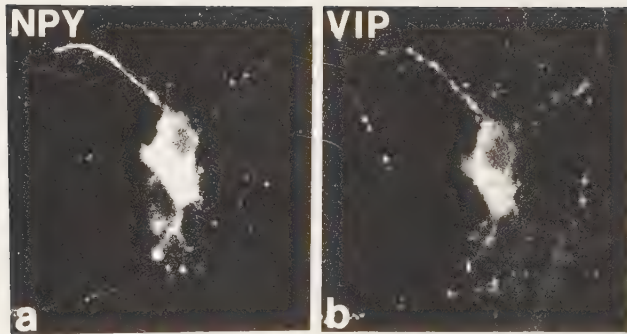


Fig. 4. Myenteric ganglion in rat jejunum. Whole mount preparation, first immunostained with the NPY antiserum (a), and after elution of the NPY antibodies immunostained for VIP (b). Note coexistence of NPY and VIP immunoreactivity in the same nerve cell body and nerve fibers. $\times 300$.

Fig. 3. Rat jejunum. Cryostat sections. Numerous VIP (a) and NPY (b) nerve fibers in the mucosa, submucosa, smooth muscle intramural ganglia. (c) CGRP nerve fibers were moderate in number in the mucosa, submucosa and intramural ganglia but few in the smooth muscle layers. (d) Somatostatin fibers were moderate in number in the submucosa and basal parts of the mucosa and in the intramural ganglia. Scattered somatostatin fibers could be found in the smooth muscle layers. (a-d) $\times 125$

Fig. 5. VIP nerve fibers and cell bodies in rat jejunum, 10 days after myectomy. Whole mounts of longitudinal smooth muscle with adherent myenteric ganglia. No loss of VIP fibers orally to the lesion. Up to 2 mm anally to the lesion the number of VIP fibers were markedly reduced. Further distally the nerve fiber density returned to normal. The occurrence and distribution of VIP nerve cell bodies were not affected by the lesion. $\times 225$

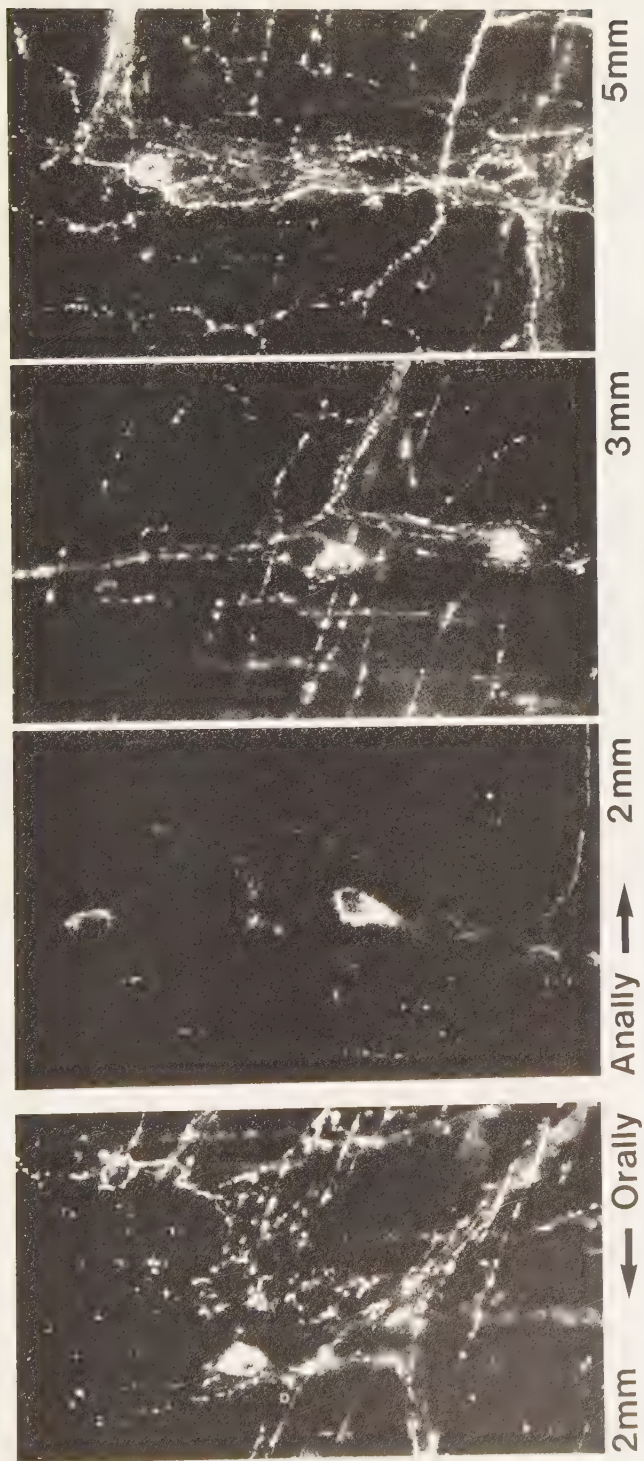


Fig.5

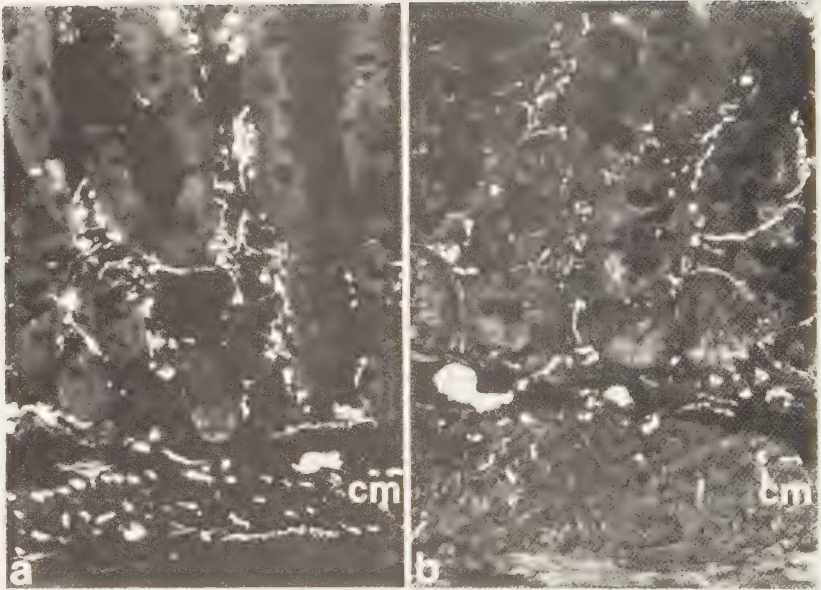


Fig. 6. Rat jejunum. Cryostat sections stained with VIP antiserum. Comparison of nerve fiber density in the intact wall (a) and in the area underlying myectomy, 10 days postoperatively (b). Note markedly reduced number of VIP fibers in the circular smooth muscle (cm) but unchanged VIP fiber density in the mucosa and submucosa. $\times 150$

Fig. 8. NPY nerve fibers and cell bodies in rat jejunum, 10 days after intestinal clamping. Cryostat sections, mucosa and submucosa visible. No loss of NPY fibers could be demonstrated in the anal direction. A marked loss of NPY fibers could be seen in the first mm orally to the lesion. Further orally the NPY fibers gradually increased in density. At 4 mm orally the frequency of NPY fibers was normal $\times 200$

Fig. 9. SP nerve fibers in rat jejunum, 10 days after myectomy. Whole mounts of longitudinal muscle with adherent myenteric ganglia. No loss of SP fibers orally to the lesion. SP fibers were lacking for 4 mm anally to the lesion. Beyond this point there was a gradual return and at 6–7 mm anally the innervation was back to normal. $\times 230$.

Fig. 10. SP nerve fibers in rat jejunum, 10 days after intestinal clamping. Cryostat sections, mucosa and submucosa visible. Both orally and anally there was a loss of SP fibers for approximately 4 mm. At 5 mm in both directions the innervation was back to normal. $\times 175$.

Fig. 11. CGRP nerve fibers in rat jejunum, 10 days after myectomy. Whole mounts of longitudinal muscle with adherent myenteric ganglia. A reduction of the number of CGRP fibers could be seen for a distance of 1–2 mm in both oral and anal direction. Further orally and anally the nerve fiber density was normal $\times 200$

Fig. 12. CGRP nerve fibers in rat jejunum 10 days after intestinal clamping. Cryostat sections, mucosa and submucosa visible. No loss of CGRP fibers anally to the lesion. CGRP fibers disappeared orally for approx. 2 mm in the submucous ganglia and for approx. 4 mm in the mucosa and submucosa. Beyond these points CGRP fibers increased gradually in number. At 3–4 mm in the submucous ganglia and at 7–8 mm in the mucosa and submucosa the number of CGRP fibers was back to normal. $\times 150$.

Fig. 13. Somatostatin nerve fibers in rat jejunum 10 days after myectomy. Whole mounts of longitudinal muscle with adherent myenteric ganglia. Orally to the lesion the number of somatostatin fibers was normal. Anally to the lesion somatostatin fibers disappeared for 2–3 mm. Beyond this point the number of fibers gradually increased and was back to normal at approx 6 mm anally to the lesion. $\times 220$.

Fig. 14. Somatostatin nerve fibers in rat jejunum 10 days after intestinal clamping. Cryostat sections, mucosa and submucosa visible. Somatostatin fibers disappeared for approx. 3 mm in both directions from the lesion. Beyond these points there was a gradual increase and a normal frequency of somatostatin fibers could be seen at 4–5 mm both orally and anally to the lesion. $\times 200$.

Fig. 15. Enkephalin nerve fibers in rat jejunum 10 days after myectomy. Whole mounts of longitudinal muscle with adherent myenteric ganglia. No loss of enkephalin fibers anally to the lesion. There was a loss of fibers for 3 mm orally to the lesion. Further orally the number of enkephalin fibers increased gradually and was back to normal at 6–7 mm. $\times 225$

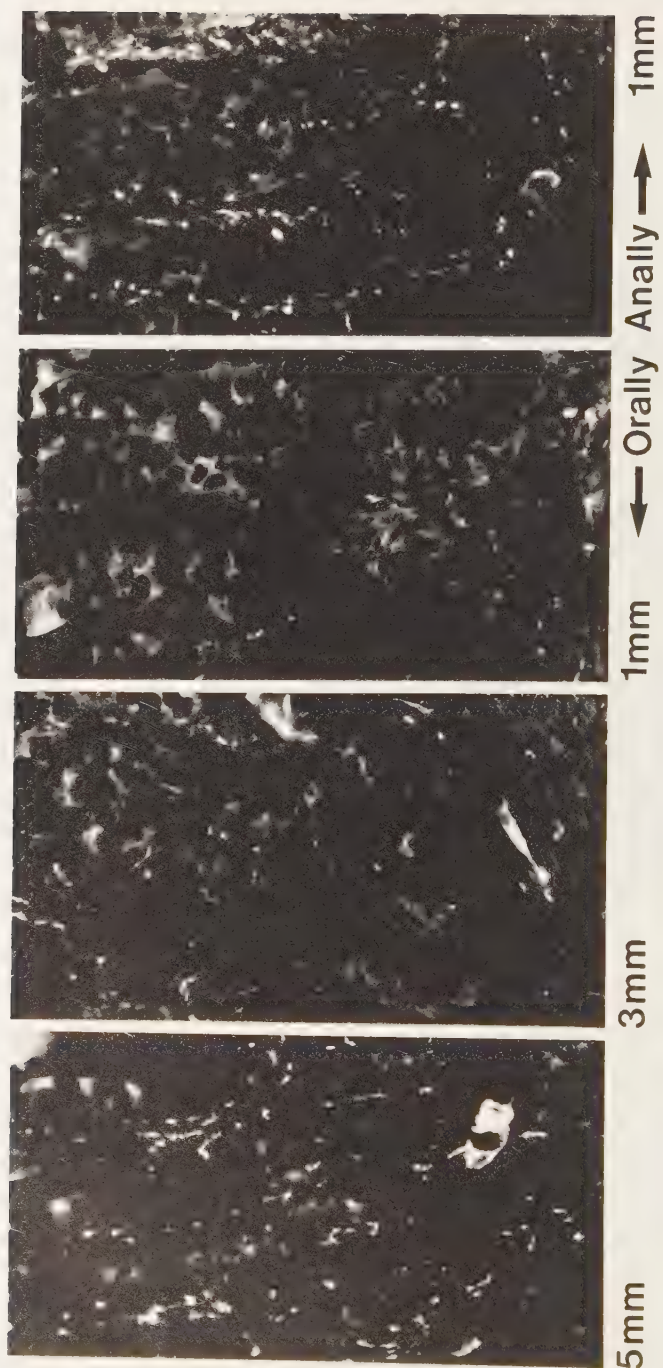


Fig.8

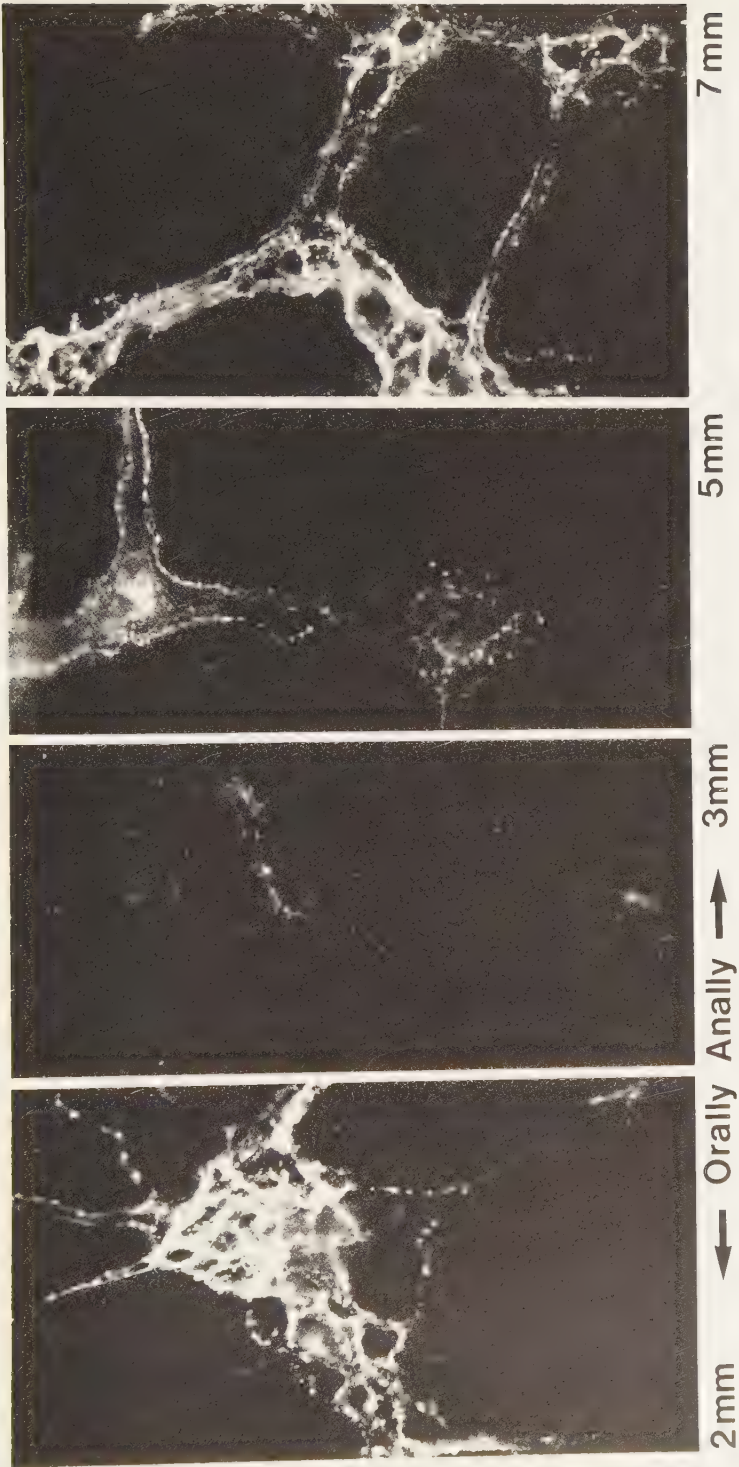


Fig.9

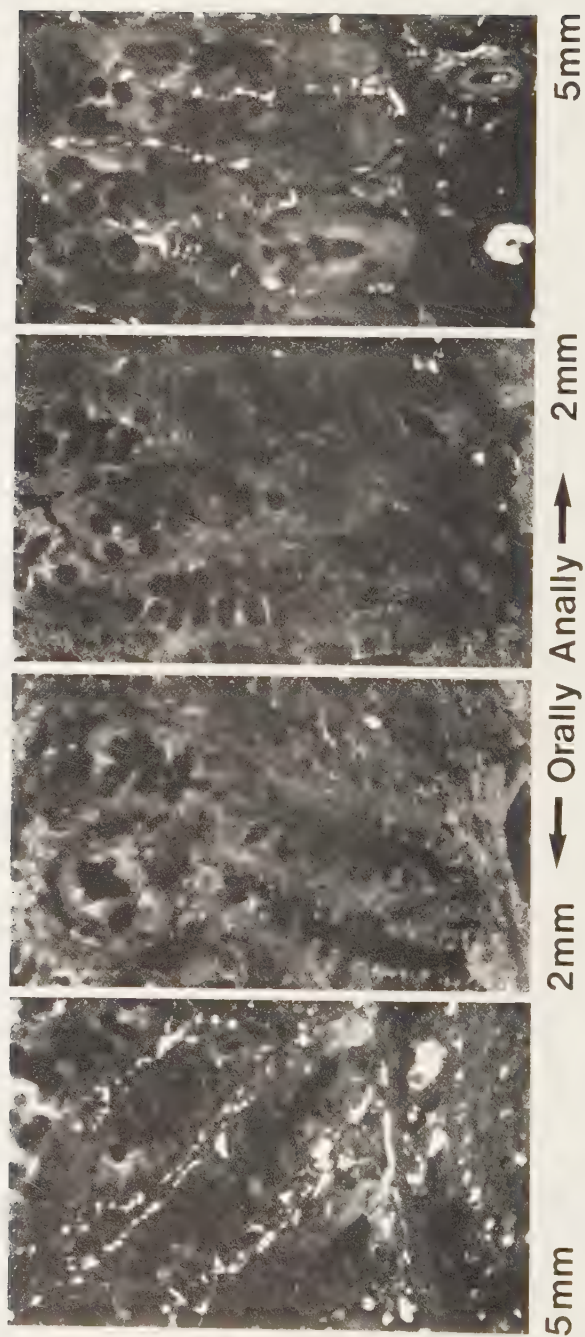


Fig.10

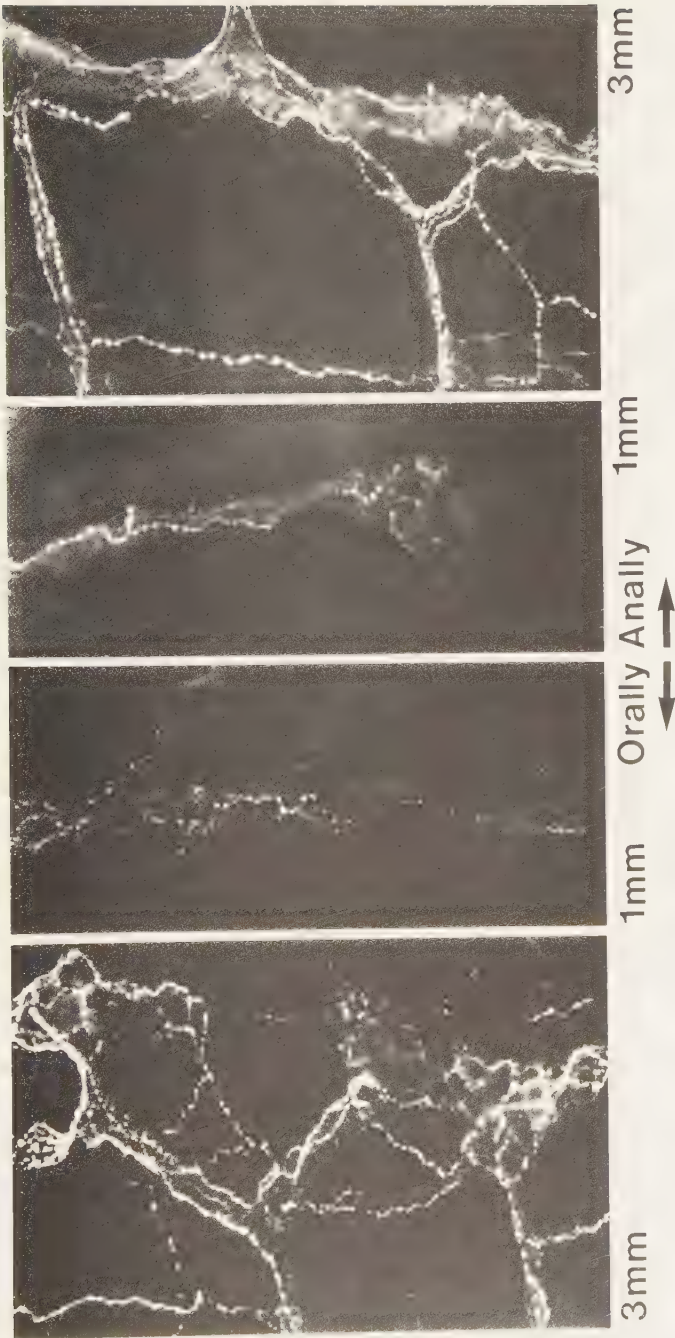


Fig.i1

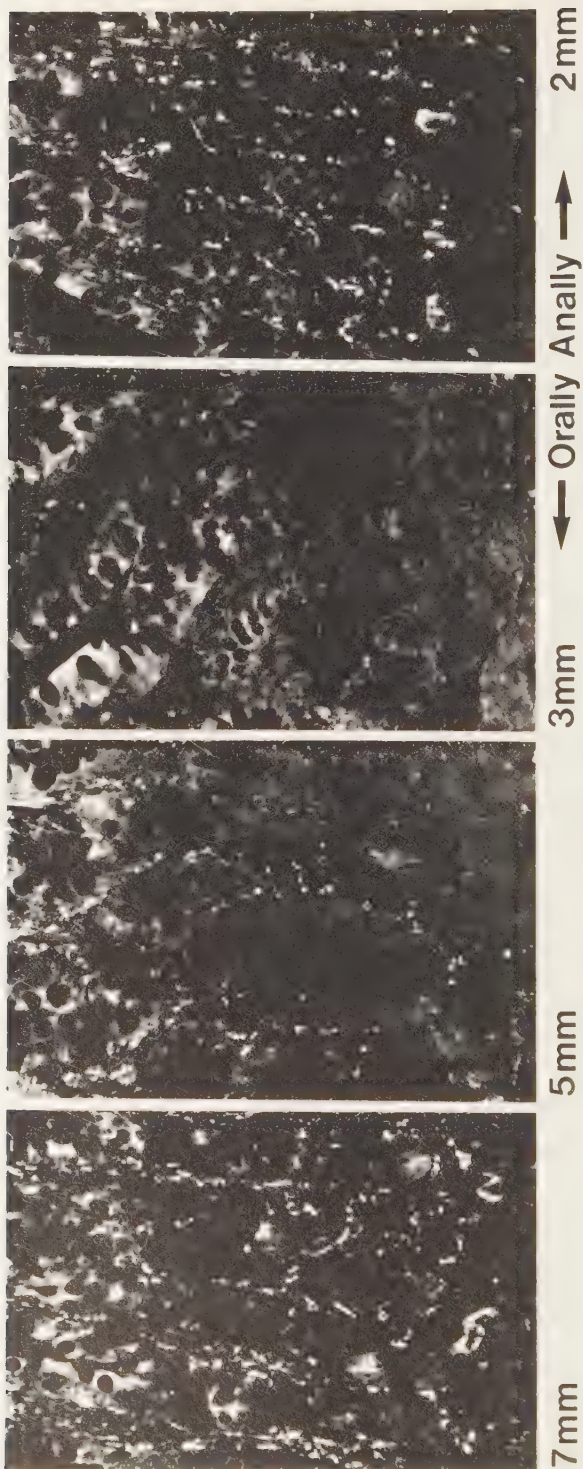


Fig.12

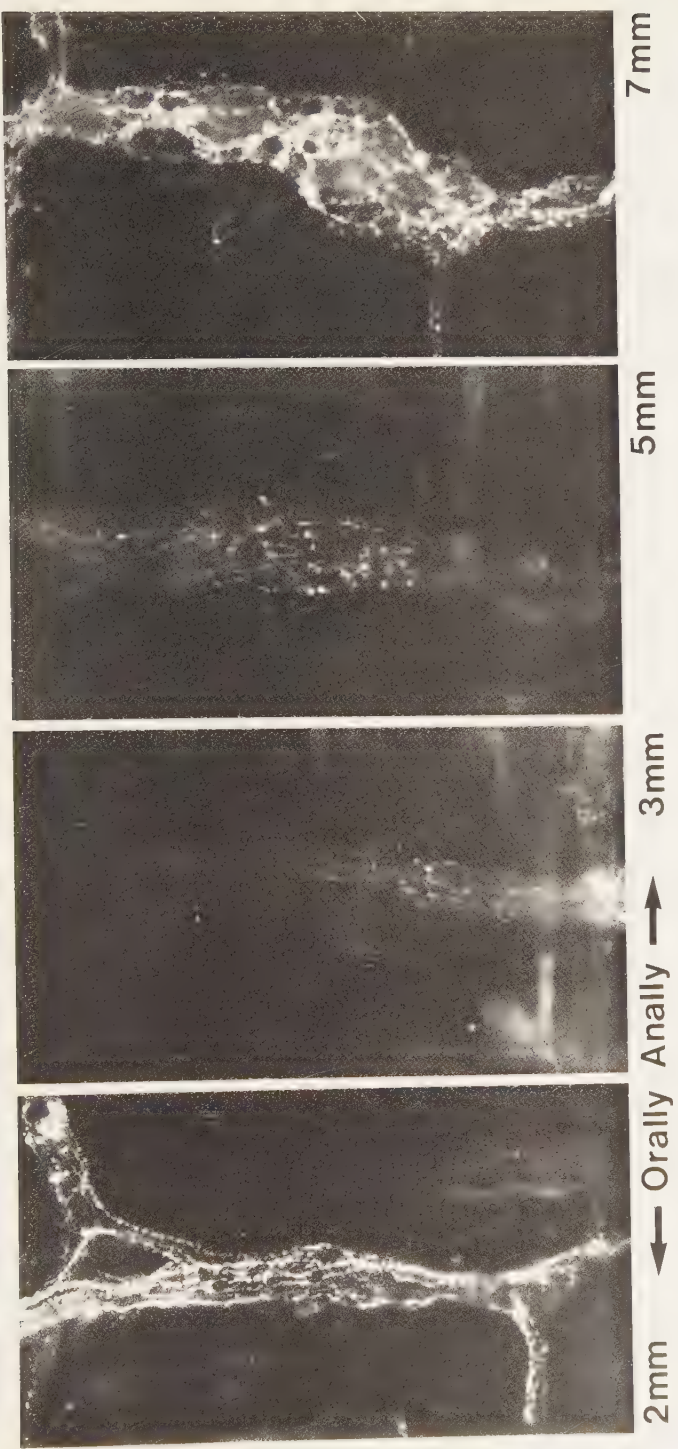


Fig.13

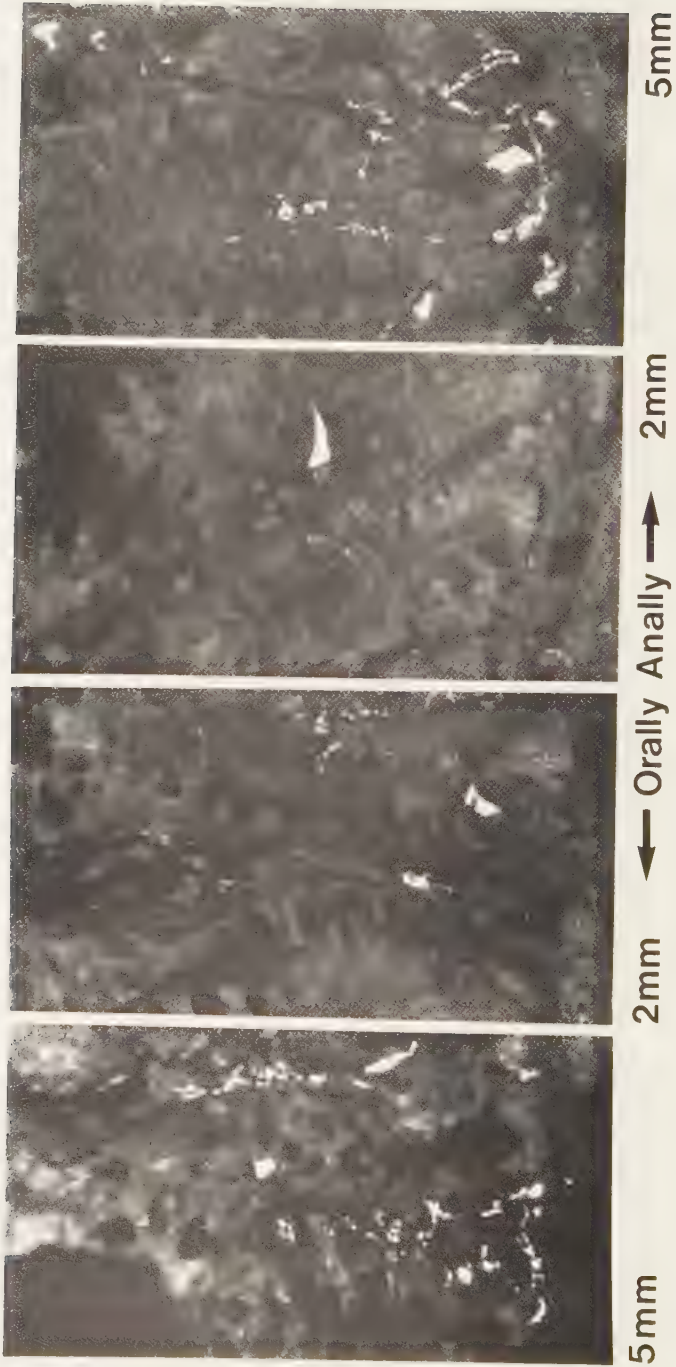


Fig.14

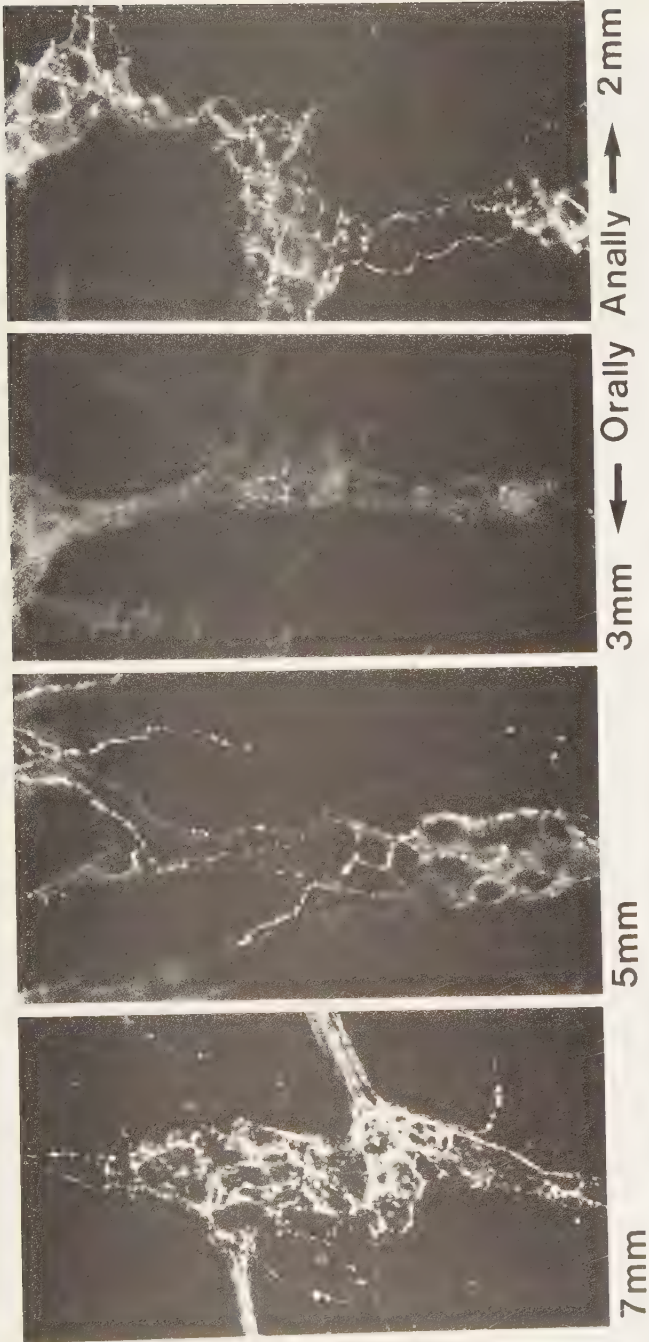
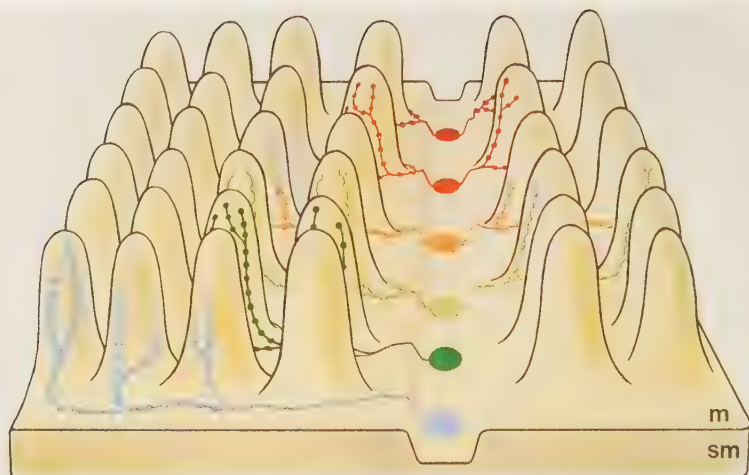
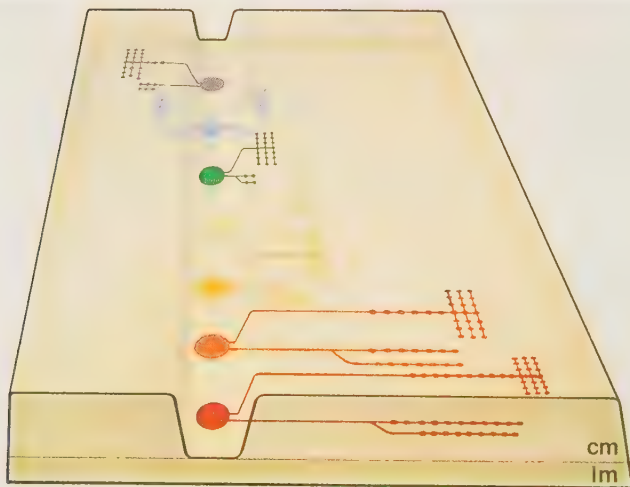


Fig.15



ORALLY ← → ANALLY

GRP Galanin Som SP VIP/NPY CGRP



ORALLY ← → ANALLY

Enk CGRP VIP/NPY SP Som Galanin GRP

Fig 16. Schematic illustration attempting to outline the terminations and projections of the different peptide-containing neurons in rat jejunum. (a) Nerve fibers emanating from the submucosal ganglia and terminating within the ganglia and in the mucosa (m) and submucosa (sm). (b) Nerve fibers emanating from the myenteric ganglia and terminating within the ganglia and longitudinal smooth muscle (lm) (straight course in figure) and in the circular smooth muscle (cm) (bent course in figure). The actual course of the fibers from the cell bodies to the termination area is unknown.

Projections of peptide-containing neurons in rat colon

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Short title: Projections of enteric neurons

Key words: Neuropeptides, enteric neurons, neuronal projections, vasoactive
intestinal peptide, calcitonin gene-related peptide, gastrin
releasing peptide, neuropeptide Y, substance P, enkephalin.

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Abstract

The distribution, origin and projections of nerve fibers containing vasoactive intestinal peptide, substance P, neuropeptide Y, galanin, gastrin releasing peptide, calcitonin gene-related peptide or enkephalin were studied in the rat large intestine by immunocytochemistry and immunochemistry. Most of these nerve fibers had an intramural origin as was established by extrinsic denervation (severing of mesenterial nerves). The polarities and projections of various peptide-containing intramural neuronal populations were studied after axonal degeneration upon local disruption of enteric nervous pathways (myectomy or intestinal clamping). Myenteric neurons containing vasoactive intestinal peptide, galanin, gastrin releasing peptide, calcitonin gene-related peptide or neuropeptide Y/vasoactive intestinal peptide gave off 5-10 mm long descending projections while those containing substance P or met-enkephalin issued approx. 5 mm long ascending projections. Submucous neurons containing calcitonin gene-related peptide or gastrin releasing peptide issued both ascending (2-6 mm) and descending (2-6 mm) projections, those containing vasoactive intestinal peptide issued ascending (approx. 2 mm) projections, while those containing galanin or neuropeptide Y/vasoactive intestinal peptide did not seem to give off oro-anal projections. Enkephalin fibers could not be detected in the mucosa and the substance P nerve fibers were too few to allow establishment of projections.

Numerous populations of peptide-containing nerves fibers have been demonstrated throughout the digestive tract. Several neuropeptides, such as vasoactive intestinal peptide (VIP), substance P (SP), enkephalin and galanin are thought to play important roles in the control of secretory and motor activities in the gut (for reviews see 5,15,24 and 28). Many studies have dealt with the intramural organization of the various populations of peptide-containing fibers in the small intestine (6,7,11,12,14,16,17,21,22,25,29), while the large intestine has attracted little interest in this respect (22).

The present study examines the distribution, origin and projection pattern of nerve fibers storing immunoreactive VIP, SP, neuropeptide Y (NPY), galanin, gastrin releasing peptide (GRP), calcitonin gene related peptide (CGRP) and enkephalin in the rat colon.

EXPERIMENTAL PROCEDURES

Animals.

70 adult female Sprague-Dawley rats (body wt 150-200 g, Alab, Stockholm, Sweden) were used. The rats were given a standard pellet food and water ad libitum.

Chemicals

Synthetic VIP, NPY, met-enkephalin, SP, galanin, GRP and CGRP were purchased from Peninsula, Belmont, CA., USA.

Denervation procedures

Severing of extrinsic nerves. Five rats were subjected to extrinsic denervation of an 6-8 cm segment of the colon by clamping mesenterial blood vessels and surrounding mesentery for 10 minutes with a pair of forceps ("nerve crush"), a technique previously used for the small intestine (12,14). The animals were left for 10 days after the operation. To confirm elimination of sympathetic fibers the tissue specimens were examined immunocytochemically using an anti-serum to dopamine β -hydroxylase (DBH), a specific marker of adrenergic neurons (27). The DBH antiserum (Eugene Tech, Allendale, N.J., USA) was used in dilution 1:320 and has previously been used for a similar purpose (20).

Severing of intrinsic nervous pathways a) Twenty rats were subjected to myectomy i.e. circumferential removal of 10 mm of the outer longitudinal smooth muscle layer together with adherent myenteric ganglia at the level of the transverse colon as previously described for the small intestine (12,14). b) Forty rats were subjected to local disruption of enteric nerve fiber pathways

by circumferential clamping of a 3-4 mm segment of the transverse colon for 10 min with a pair of forceps as previously described for the jejunum (12). All animals were killed 7-10 days after surgery.

The myectomy severed the nervous pathways connecting the myenteric ganglia (leaving the submucous ganglia unaffected) and eliminated also the extrinsic innervation. It cannot be excluded that some myenteric neurons remained attached to the circular muscle after the myectomy. Such retained nerve cell bodies could not be detected by immunocytochemistry, however.

The intestinal clamping severed both myenteric and submucous pathways.

Immunocytochemistry

Tissue specimens were collected from the transverse colon of unoperated and operated rats. In the animals with severed enteric nervous pathways one segment (2-3 cm) was taken orally and one anally to the lesion. The specimens were opened along the mesenteric border. In order to avoid tissue shrinkage due to fixation all specimens were pinned flat, with a minimum of stretching, on a piece of balsa wood with the mucosal side facing the wood. The segments were orientated in such a way that the oral and anal ends could be identified. The specimens were fixed overnight by floating in a mixture of 2% formaldehyde and 15% of a saturated aqueous picric acid solution in 0.1 M phosphate buffer (pH 7.2) and thoroughly rinsed in Tyrode solution containing 10% sucrose. They were processed for immunocytochemistry either as whole mounts (13) or as cryostat sections. The whole mounts consisted of one of the following three layers: the outer (longitudinal) muscle layer, the inner (circular) muscle layer and the submucosa. The whole mounts were placed on chrome-alum-coated slides; before further processing they were dried, frozen and thawed in order to facilitate antibody penetration (12). For cryostat sectioning specimens were frozen on dry ice and longitudinal sections were cut at 10 μ m thickness. Sections and whole mounts were processed for the immunocytochemical demonstration of VIP, NPY, SP,

galanin, GRP, enkephalin and CGRP using previously characterized rabbit antisera (Table 1) and the indirect immunofluorescence method of Coons et al (4). In order to reveal co-existence of VIP and NPY in the same nerve fibers, sections and whole mounts were first immunostained for NPY or VIP, examined in a fluorescence microscope and photographed. The antibodies were then eluted with acid potassium permanganate for 30-60 s (32). The completeness of the elution was tested by applying fluoresceinated anti-rabbit IgG. Sections devoid of immunofluorescence were then processed for the immunocytochemical demonstration of VIP or NPY and again photographed. In order to reveal co-existence of CGRP and SP or CGRP and VIP, sections and whole mounts were stained simultaneously with a guinea pig antiserum against CGRP (code no 8513, raised against unconjugated synthetic CGRP, Milab, Malmö, Sweden) and a rabbit antiserum against SP or VIP (Table 1). The site of the antigen-antibody reaction was visualized in the case of CGRP by fluorescein isothiocyanate (FITC)-conjugated antibodies to guinea pig IgG raised in goat (Sigma, St. Louis, Mo), and in the case of SP or VIP by tetramethylrhodamine isothiocyanate (TRITC)-conjugated antibodies to rabbit IgG raised in pig (Milab, Malmö, Sweden). Filters (Zeiss no. 09 and 15) were shifted to enable selective excitation (at 485 and 546 nm) of the two fluorophores. For controls, antisera that had been inactivated by the addition of an excess amount of antigen (10-100 ug of synthetic peptide/ml diluted antiserum) were used. Cross-reaction with other peptides or proteins containing amino acid sequences recognized by the different antisera cannot be excluded. It is appropriate, therefore, to refer to the immunoreactive material as VIP-like, NPY-like and so on. For simplicity, however, the shorter terms are used in the following.

Estimation of nerve fiber length

The fiber length of each neuronal population was established, after axonal degeneration upon severing of intrinsic enteric nervous pathways, by measuring the distance from the lesion to the region showing a normal fiber frequency. This distance comprised a region lacking nerve terminals (this region contains nerve cell bodies and preterminals with a peptide content at or below the detection limit of the technique) and a region displaying a gradual increase in the number of terminals (these terminals derive from nerve cell bodies located immediately adjacent to the lesion) (c.f. 12). Distances were measured using a micrometer scale inserted in the visual field. Both cryostat sections and whole mount preparations (at least 6 of each) were used to establish the projection distance of each of the nerve fiber populations.

Radioimmunochemistry

The concentrations of CGRP, met-enkephalin, SP and GRP in the transverse colon were determined by radioimmunoassay (RIA). Gut segments were opened, rinsed and cut serially in 5 mm specimens, 5 orally and 5 anally to the lesion. The specimens were weighed and boiled in 0.5 M acetic acid (0.1 g wet weight/ml) for 15 min and homogenized (Polytron) for 1-2 min. After centrifugation at 2000 g for 15 min the supernatants were collected and lyophilized. The material was freeze-dried and taken up in 2 ml of 0.05 M phosphate buffer (pH 7.4) containing 0.25% human serum albumin.

For RIA of CGRP we used a rabbit antiserum (code no. A13, Milab, Malmö, Sweden) raised against albumin-conjugated synthetic rat CGRP. The antiserum was used in a final dilution of 1:30000. The detection limit was 20 pmol/l. There was no cross-reaction with human calcitonin, katacalcin or calcitonin C-terminal adjacent peptide (see also 19). The interassay variation was below 11% in the range 100-300 pmol/l. In the met-enkephalin RIA we used a rabbit antiserum (R-26, a kind gift from prof. K. Voigt. Freie Universität, Berlin) in a final

dilution of 1:20000 (33). The detection limit was 10 pmol/l. The interassay variation was below 10% in the range 50-250 pmol/l. The antibody which recognizes the C-terminus of met-enkephalin did not cross-react with leu-enkephalin, β -endorphin (1-31), β -endorphin (1-17), β -endorphin (1-9), met-enkephalin-Arg⁶-Phe⁷, dynorphin (1-8), BAM 12P (bovine adrenal medulla dodecapeptide) or peptide E (33). Immunoreactive SP was determined using a rabbit antiserum raised against synthetic SP (code no. SP2, a kind gift from Dr. E. Brodin, Stockholm, Sweden). The SP RIA has previously been described in detail (3). The detection limit was 10 pmol/l and the interassay variation was 9% in the range 20-90 pmol/l. The antiserum did not detect CGRP or any known tachykinins besides SP. For RIA of GRP we used a bombesin antiserum (code no. 8050, Milab, Malmö, Sweden) that crossreacted with GRP (30%) and GRP 14-27 (37%) under the assay conditions (26). The antiserum was used in dilution 1:14000 and the interassay variation was 11% in the range 20-400 pmol/l. The concentrations were expressed as pg porcine GRP equivalents per g wet weight. Details of the GRP RIA have been given elsewhere (26).

RESULTS AND COMMENTS

All the antisera demonstrated neuronal elements in the colon of the rat. Each neuronal population had its own characteristic topographic distribution, summarized in Figs. 1 and 2. In agreement with earlier findings in the rat small intestine (8,12) VIP and NPY were found to coexist; in the colon however, only a small proportion of the VIP fiber population harboured NPY. In addition, NPY occurred in perivascular fibers which probably contain noradrenaline since they stained with an antiserum against DBH (Ekblad, unpublished) (see also 9,10 and 39). As in the rat small intestine (12) SP and CGRP in the colon occurred in separate nerve fiber populations in the smooth muscle and mucosa while coexisting in a population of perivascular fibers (Fig. 3). Only a minor

proportion of the perivascular CGRP fiber population harboured SP, while all SP-containing perivascular fibers contained CGRP. Some perivascular CGRP fibers contained VIP instead of SP (Fig. 3).

Extrinsic denervation. Mesenteric clamping eliminated two perivascular nerve fiber populations; those that contained NPY (and probably noradrenaline) and those that contained SP/CGRP. The latter fibers are probably of sensory origin since it has been shown in several previous studies that SP and CGRP coexist in a prominent population of primary sensory neurones (c.f. 30). Other types of peptide-containing nerve fibers were not overtly affected by extrinsic denervation; this included perivascular CGRP/VIP fibers.

Disruption of enteric nervous pathways. Distribution and projections of individual enteric neuronal systems

The various neuronal systems are described in order of over-all nerve fiber frequency.

VIP neurones. Numerous fine-varicose intensely immunoreactive nerve fibers were observed throughout the intestinal wall, including the intramural ganglia which harboured in addition a great number of immunoreactive nerve cell bodies (Figs. 1 and 2). A moderate number of VIP fibers could be detected around submucosal arteries and veins; some of these perivascular fibers contained also CGRP (Fig. 3). After myectomy very few VIP fibers remained in the underlying circular muscle; the frequency of VIP fibers in the mucosa and submucosa was not altered. No loss of VIP fibers could be detected in the muscle layers and myenteric ganglia orally to the myectomy or the intestinal clamping; anally to the lesion there was a loss of VIP fibers for 1-2 mm and a gradual increase in the number of fibers up to 4 mm: beyond this point the fiber frequency was back to normal. Intestinal clamping produced a marked reduction of VIP fibers in the mucosa and

submucosa up to 2 mm orally to the lesion, further orally the fiber frequency was normal. There was no loss of fibers in the mucosa or submucosa anally to the lesion (Fig. 5). Thus, myenteric VIP neurons issue descending projections, approx. 3 mm in length, that terminate within the ganglia and the smooth muscle. VIP fibers emanating from the submucous ganglia project in oral direction for approx. 2 mm to other ganglia and to the mucosa and submucosa. Some VIP fibers from submucous ganglia probably terminate in the circular muscle layer (since a few VIP fibers remained in this location after myectomy).

Galanin neurons. Fine-varicose, intensely immunoreactive galanin nerve fibers were numerous in the myenteric and submucous ganglia. A few fibers occurred in the smooth muscle, in the mucosa and around blood vessels. The submucosa harboured a moderate number of galanin fibers. Moderately immunoreactive nerve cell bodies were seen in submucous as well as myenteric ganglia (Figs. 1 and 2). Myectomy caused a loss of galanin fibers in the underlying circular muscle but did not affect the number of galanin fibers in the mucosa, submucosa, submucous ganglia or around blood vessels (Fig. 4). Myectomy or intestinal clamping resulted in a loss of galanin fibers in myenteric ganglia and smooth muscle up to 3 mm anally to the lesion. The fibers gradually increased in frequency over a distance of 3-6 mm further anally; beyond this point there was a normal number of galanin fibers. Orally to the lesion galanin fibers occurred with a normal frequency in the myenteric ganglia and muscle layers (fig. 6). Intestinal clamping did not produce any obvious change in the number of galanin fibers in the mucosa or submucosa (Fig. 6). Thus, myenteric galanin neurons issue descending projections, approx. 6 mm in length, that terminate in other myenteric ganglia and in the muscle layers. Galanin nerve fibers from submucous ganglia probably project towards the mucosal surface or possibly laterally since there was no loss of fibers either orally or anally to the lesion.

CGRP neurons. Fine-varicose, intensely immunoreactive CGRP fibers were numerous in the myenteric ganglia and around blood vessels and few in the muscle layers. Some perivascular CGRP fibers contained SP; these fibers have an extrinsic, probably sensory derivation. CGRP fibers were moderate in number in the mucosa, submucosa and submucous ganglia. Nerve cell bodies displaying a moderate CGRP immunoreactivity were regularly seen in the intramural ganglia (Figs. 1 and 2). Myectomy caused a loss of CGRP fibers in the underlying circular muscle layer and a reduction in the number of submucous perivascular fibers in the same segment, but there was no reduction in the number of CGRP fibers in the mucosa or submucous ganglia. In the myenteric ganglia and smooth muscle there was a loss of fibers up to 3 mm anally to the site of myectomy or intestinal clamping. From 3 to 5 mm further anally the fibers gradually increased in frequency, being back to normal at approx. 5 mm. The number of CGRP fibers in the myenteric ganglia and smooth muscle was unaffected orally to the lesion (Fig. 7). Intestinal clamping caused a loss of mucosal CGRP fibers for approx. 3 mm both orally and anally to the lesion. Beyond these points there was a gradual increase in the number of fibers up to approx. 6 mm where the fiber frequency was back to normal (Fig. 7). The concentration of CGRP was reduced both in the oral and anal segments adjacent to the lesion (Fig. 8A). Thus, myenteric CGRP neurons issue descending projections approx. 5 mm in length to myenteric ganglia and the muscle layers. Submucous CGRP neurons on the other hand give off both ascending and descending projections, 5-6 mm in length.

GRP neurons. Fine-varicose, intensely immunoreactive GRP fibers were numerous in the myenteric ganglia and few in the smooth muscle. A moderate number of GRP fibers occurred in the mucosa, submucosa and submucous ganglia. No GRP fibers could be detected around blood vessels. Moderately immunoreactive nerve cell bodies were regularly seen in the myenteric ganglia; only occasionally were they seen in the submucous ganglia (Figs. 1 and 2). Myectomy caused a loss of

GRP fibers in the underlying circular muscle; in the mucosa and submucosa they were reduced in number by approx. 50%. In the myenteric ganglia and smooth muscle GRP fibers were lacking for up to 6 mm anally to the myectomy or the intestinal clamping. From 6 to 10 mm the fibers gradually increased in number and approx. 10 mm from the lesion the fiber frequency was back to normal. Orally to the lesion there was no overt loss of myenteric GRP fibers (Fig. 9). Intestinal clamping caused a loss of GRP fibers in the mucosa and submucosa for 1-2 mm in both oral and anal direction. Further away from the lesion the fibers gradually increased in number and the frequency was back to normal approx. 3 mm from the lesion in both directions (Fig. 9). The concentration of GRP was reduced in the two segments anally to the site of intestinal clamping (Fig. 8B). Thus, myenteric GRP neurons issue descending projections approx. 10 mm in length terminating in myenteric ganglia and smooth muscle. In addition, some myenteric GRP neurons probably project to the mucosa and submucosa since myectomy greatly reduced the number of GRP fibers in these locations. Submucous GRP neurons issue both ascending and descending projections of about 2-3 mm in length.

NPY/VIP neurons. This heading includes the neuronal elements that contain NPY and VIP, i.e. the entire population of intrinsic NPY-containing neurons and a minor proportion of the VIP-containing neurons. Perivascular NPY fibers contain noradrenaline (but not VIP) and have an extrinsic, probably sympathetic derivation. NPY/VIP fibers were few in the myenteric ganglia and smooth muscle, and they were moderate in number in the mucosa, submucosa and submucous ganglia. Immunoreactive nerve cell bodies were occasionally detected in the myenteric and submucous ganglia (Figs. 1 and 2). Myectomy eliminated NPY/VIP fibers from the underlying circular muscle without affecting the number of fibers in the mucosa and submucosa. In the myenteric ganglia and smooth muscle NPY/VIP fibers were lacking for 1-2 mm anally to the myectomy or the intestinal clamping.

Further distally the fibers gradually increased in frequency; beyond 4 mm there was a normal number of NPY/VIP fibers. The fiber frequency orally to the lesion was not affected (Fig. 10). Intestinal clamping did not affect the number of mucosal NPY/VIP fibers either orally or anally to the lesion (Fig. 10). Thus, myenteric NPY/VIP neurons project anally for approx. 4 mm to myenteric ganglia and smooth muscle. Submucous NPY/VIP neurons do not seem to project in the oro-anal directions. They probably project towards the mucosal surface and possibly also laterally since there was no loss of mucosal fibers either orally or anally to the lesion.

SP neurons. Fine-varicose intensely immunoreactive SP fibers were numerous in the myenteric ganglia. They were few in the muscle layers and around blood vessels and rare in the mucosa, submucosa or submucous ganglia. The perivascular SP fibers are extrinsic, probably sensory in nature. Weakly immunoreactive nerve cell bodies were occasionally detected in the myenteric ganglia (Figs. 1 and 2). Myectomy caused a loss of SP fibers in the underlying circular muscle. Myectomy or intestinal clamping resulted in loss of SP fibers 2-3 mm orally to the lesion, followed by a gradual increase in the number of SP fibers up to about 5 mm where the fiber frequency was back to normal. There was no loss of fibers anally to the lesion (Fig. 11). In the mucosa and submucosa SP fibers were to a few to allow establishment of projections. The concentration of SP was reduced orally to the site of intestinal clamping (Fig. 8C). Thus, myenteric SP neurons project orally for approx. 5 mm to other myenteric ganglia and to the muscle layers.

Enkephalin neurons. A few moderately immunoreactive nerve fibers could be detected in the myenteric ganglia, but they were rare in the muscle layers. No fibers could be observed in the mucosa, submucosa or submucous ganglia. Occasionally moderately immunoreactive nerve cell bodies could be detected in the

myenteric ganglia (Fig. 1). After myectomy enkephalin nerve fibers disappeared in the underlying circular muscle. Orally to the lesion (myectomy or intestinal clamping) enkephalin fibers were lacking for approx. 3 mm. From 3 up to 5 mm from the lesion there was a gradual increase in the number of fibers. Further distally the fiber frequency was normal. There was no loss of fibers anally to the lesion (Fig. 11). The concentration of enkephalin was reduced orally to the site of intestinal clamping (Fig. 8D). This confirmed the immunocytochemical observations indicating that myenteric enkephalin neurons issue ascending projections approx. 5 mm in length, to myenteric ganglia and to smooth muscle.

DISCUSSION

The colon is rich in neuropeptides, which are stored for the most part in intramural neurons. As in the small intestine (6,7,11,12,14,16,22,23) each peptide-containing neuronal population in the colon seems to have its own characteristic distribution and projection pattern. There are interesting differences between the small and large intestines with respect to the projection patterns of the different neuronal populations and also with respect to the observed constellations of coexisting neuropeptides (8,12). The entire population of VIP-immunoreactive intramural fibers in the rat small intestine contains immunoreactive NPY while in the colon only a minor proportion of the VIP neurons harboured NPY. Further, the entire population of perivascular CGRP fibers in the small intestine contains SP (12,18,30) whereas in the large intestine only a small proportion of the CGRP fibers harboured SP. In fact, some perivascular CGRP fibers in the large intestine contained VIP instead of SP.

In the rat large intestine myenteric VIP, galanin, GRP, CGRP and NPY/VIP neurons gave off descending projections while SP and met-enkephalin neurons issued ascending projections. This is in accordance with findings in the rat small intestine (6,7,11,12,14,17,22) except for the CGRP neurons which issue

both ascending and descending projections in the rat small intestine (12) and the SP neurons which issue descending projections in the small intestine (12). The average lengths of the projections of the different myenteric neurons in the colon agreed rather well with those previously found in the small intestine (12,14,18,22), although the projections of the myenteric GRP and galanin neurons were shorter (10 and 6 mm respectively) in the colon than in the small intestine (20 and 15 mm respectively) (6,7,11). This may suggest that the reflex arcs are shorter in the large intestine than in the small intestine possibly explaining the differences in transit time between the large and small intestine. In the rat colon submucous CGRP and GRP neurons issued both ascending and descending projections, VIP neurons were ascending, while submucous galanin and NPY/VIP neurons did not show any oro-anal projections. In the rat small intestine submucous NPY/VIP and CGRP neurons issue ascending projections, while SP, somatostatin, galanin and GRP neurons project in both directions (12).

Taken together the results of the present study indicate that intramural peptide-containing neurons in the rat large intestine are arranged in principle as in the small intestine but that they may differ with respect to peptide content, polarities and projection distances. The functional significance of these morphological findings is still an open question. No doubt, knowledge of the neuronal circuits and the transmitters employed by the various components of these circuits will help us understand the mechanisms behind colonic activities such as peristalsis, water reabsorption and mucus secretion which are known to be regulated to a very great extent by local nerve reflexes.

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References

1. Alumets J., Håkanson R., Sundler F. and Chang K-J. (1978) Leu-enkephalin-like material in nerves and enterocromaffin cells in the gut. *Histochemistry* 56,187-196.

2. Brodin E., Alumets J., Håkanson R., Leander S, and Sundler F. (1981) Immunoreactive substance P in the chicken gut: distribution, development and possible functional significance. *Cell Tissue Res.* 216,455-469.

3. Brodin E., Lindefors N., Theodorsson-Norheim E., Peterson L., Bartfai T., Ögren S-O. and Rosell S. (1985) Tachykinins in rat central nervous system: Distribution, molecular forms, release and effects of chronic treatment with antidepressant drugs. In. *Tachykinin Antagonists. Proc. Fernström Symp., Vol 6* (eds Håkanson R. and Sundler F.) pp 3-14 Elsevier, Amsterdam.

4. Coons A.H., Leduc E.H. and Connolly J.M. (1955) Studies on antibody production I. A method for the histochemical demonstration of specific antibody and its application to a study of the hyperimmune rabbit. *J. Exp. Med.* 102,49-60.

5. Costa M. and Furness J.B. (1982) Nervous control of intestinal motility. In: *Handbook of Experimental Pharmacology* (ed. G. Bertaccini) Springer-Verlag, Berlin, Germany. vol 59 p 279-382.

6. Costa M., Furness J.B., Yanaihara N., Yanaihara C. and Moody T.W. (1984) Distribution and projections of neurons with immunoreactivity for both gastrin-releasing peptide and bombesin in the guinea-pig small intestine. *Cell Tissue Res.* 235,285-293.

7. Ekblad E., Ekman R., Håkanson R. and Sundler F. (1983) GRP neurones in the rat small intestine issue long anal projections. *Regul. Pept.* 9,279-287.
8. Ekblad E., Håkanson R. and Sundler F. (1984) VIP and PHI coexist with an NPY-like peptide in intramural neurones of the small intestine. *Regul. Pept.* 10,47-55.
9. Ekblad E., Wahlestedt C., Ekelund M., Håkanson R. and Sundler F. (1984) Neuropeptide Y in the gut and pancreas: distribution and possible vasomotor function. *Front. Horm. Res.* 12,85-90.
10. Ekblad E., Ekelund M., Graffner H., Håkanson R., Sundler F. Peptide-containing nerve fibres in the stomach wall of rat and mouse. *Gastroenterology* 89:73-85, 1985
11. Ekblad E., Rökaeus Å., Håkanson R. and Sundler F. (1985) Galanin nerve fibers in the rat gut: distribution, origin and projections. *Neuroscience* 16,355-363.
12. Ekblad E., Winther C., Ekman R., Håkanson R. and Sundler F. (1986) Projections of peptide-containing neurons in rat small intestine. *Neuroscience* 20,000-000.
13. Furness J.B. and Costa M. (1978) Distribution of intrinsic nerve cell bodies and axons which take up aromatic amines and their precursors in the small intestine of the guinea pig. *Cell Tissue Res.* 188,527-543.

14. Furness J.B., Costa M., Llewellyn-Smith I.J., Franco R. and Wilson A.J.
(1981) Polarity and projections of peptide-containing neurons in the guinea pig small intestine. In: Cellular Basis of Chemical Messengers in the Digestive System, (eds. Grossman M.I., Brazier M.A.B. and Lechago J.) Acad. Press, New York no. 23 pp. 201-213.
15. Furness J.B. and Costa M. (1982) Identification of gastrointestinal neurotransmitters. In: Handbook of Experimental Pharmacology, (ed. Bertaccini G.) Springer-Verlag, Berlin, vol. 59 p. 383-460.
16. Furness J.B., Costa M., Emson P.C., Håkanson R., Moghimzadeh E., Sundler F., Taylor I.L. and Chance R.E. (1983) Distribution, pathways and reactions to drug treatment of nerves with neuropeptide Y- and pancreatic polypeptide-like immunoreactivity in the guinea-pig digestive tract. Cell Tissue Res. 234,71-92.
17. Furness J.B., Costa M. and Miller R.J. (1983) Distribution and projections of nerves with enkephalin-like immunoreactivity in the guinea-pig small intestine. Neuroscience 8,653-664.
18. Gibbins I.L., Furness J.B., Costa M., MacIntyre I., Hillyard C.J. and Girgis S. (1985) Co-localization of calcitonin gene-related peptide-like immunoreactivity with substance P in cutaneous, vascular and visceral sensory neurons of guinea pigs. Neurosci. Lett. 57,125-130.
19. Grunditz T., Ekman R., Håkanson R., Rerup C., Sundler F. and Uddman R. Calcitonin gene-related peptide in the thyroid gland: Distribution and effects on thyroid hormone secretion. Endocrinology. In press.

20. Grunditz T., Håkanson R., Sundler, F. and Uddman, R. (1987) Neuronal pathways to the rat thyroid revealed by retrograde tracing and immunocytochemistry. *Neurosci.*, submitted.
21. Keast J.R., Furness J.B. and Costa M. (1984) Origins of peptide and norepinephrine nerves in the mucosa of the guinea pig small intestine. *Gastroenterology* 86,637-644.
22. Kobayashi S. and Nishisaka T. (1985) Myenteric enkephalin neurons around the Laser-photo-coagulation necrosis: an immunocytochemical investigation in the guinea pig jejunum and proximal colon. *Arch. histol. jap.* 48,239-254.
23. Kosterlitz H.W. and Watt A.J. (1975) The peristaltic reflex. In: *Methods in Pharmacology* (eds. Daniel E.E. and Paton D.M.) Plenum, New York vol 3 p. 391-401.
24. Makhlof G.M. (1985) Enteric neuropeptides: role in neuromuscular activity of the gut. *Trends in Pharm. Sci.* 6,214-218.
25. Malmfors G., Leander S., Brodin E., Håkanson R., Holmin T. and Sundler F. (1981) Peptide containing neurones intrinsic to the gut wall. An experimental study in the pig. *Cell Tissue Res* 214,225-238.
26. Moghizadeh E., Ekman R., Håkanson R., Yanaihara N. and Sundler F. (1983) Neuronal gastrin-releasing peptide in the mammalian gut and pancreas. *Neuroscience* 10,553-563.

27. Rush R.A. and Geffen L.B. (1981) Dopamine β -hydroxylase in health and disease. *CRC Crit. Rev. Clin. Lab. Sci.* 12,241-277.
28. Sundler F., Håkanson R. and Leander S. (1980) Peptidergic nervous systems in the gut. *Clin. Gastroenterol.* 9,517-543.
29. Sundler F., Moghimzadeh E., Håkanson R., Ekelund M. and Emson P. (1983) Nerve fibers in the gut and pancreas of the rat displaying neuropeptide-Y immunoreactivity. Intrinsic and extrinsic origin. *Cell Tissue Res.* 230,487-493.
30. Sundler F., Brodin E., Ekblad E., Håkanson R. and Uddman R. (1985) Sensory nerve fibers: Distribution of substance P, neurokinin A and calcitonin gene-related peptide. In: *Tachykinin Antagonists. Proc. Fernström Symp.* (eds. Håkanson R. and Sundler F.) Elsevier, Amsterdam, vol. 6. p. 3-14.
31. Sundler F., Ekblad E., Böttcher G., Alumets J. and Håkanson R. (1985) Coexistence of peptides in the neuroendocrine system. In: *Biogenetics of Neurohormonal Peptides.* (eds. Håkanson R and Thorell J) Acad. Press London. p. 213-243.
32. Tramu G., Pillez A. and Leonardelli J. (1980) An efficient method of antibody elution for the successive or simultaneous location of two antigens by immunocytochemistry. *J. Histochem. Cytochem.* 26,322-324.
33. Voigt K.H., Weber E. and Martin R. (1980) Neuropeptides, subcellular localization of ACTH and related peptides. In: *Structure and Activity of Natural Peptides* (eds. Voelter W and Weitzel G), de Gruyter, Berlin. 115-140.

Legends to figures

- Fig. 1. Schematic outline of the topography and relative density of nerve fibers (—) and nerve cell bodies (O) in the rat colon. SG, submucous ganglia; cm, circular muscle; MG, myenteric ganglia; lm, longitudinal muscle.
- Fig. 2. Rat colon, cryostat sections. VIP fibers were numerous throughout the wall. Galanin fibers were numerous in the intramural ganglia and in the submucosa and few in the muscle layers and mucosa. CGRP fibers were numerous in the myenteric ganglia and moderate in number in the submucosa and mucosa, they were conspicuous at the mucosal surface. GRP fibers were numerous in the myenteric ganglia and moderate in number in the muscle layers, mucosa and submucosa. NPY/VIP fibers as represented here by NPY immunoreactive fibers were few in the myenteric ganglia, moderate in number in the muscle layers, mucosa and submucosa. NPY/noradrenaline fibers as represented here by NPY immunoreactive fibers were numerous around blood vessels. SP fibers were numerous in the myenteric ganglia but few in the smooth muscle, mucosa and submucosa. X100.
- Fig. 3. Submucous blood vessels in rat colon. Whole mount preparations. Double immunostaining revealed coexistence of CGRP (FITC) and SP (TRITC) (upper panel) and of CGRP (FITC) and VIP (TRITC) (lower panel), respectively. The CGRP/SP fibers were distinct from the CGRP/VIP fibers. Perivascular CGRP immunoreactive fibers were more numerous than either SP or VIP immunoreactive fibers. X200.

- Fig. 4. Rat colon. Cryostat sections stained for VIP (A and B) or galanin (C and D) for comparison of the nerve fiber frequency in intact wall (A and C) and in the myectomized segment 8 days postoperatively (B and D). Note markedly reduced number of both VIP and galanin fibers in the circular muscle but unchanged VIP and galanin fiber frequency in the mucosa and submucosa. X130.
- Fig. 5. VIP immunoreactive nerve fibers (including VIP and VIP/NPY fibers) in rat colon, 10 days after intestinal clamping. Upper panel: Cryostat sections, mucosa and submucosa visible. No loss of VIP fibers anally to the lesion. Orally there was a markedly reduced number of VIP fibers for approx. 2 mm, further orally the fiber frequency was normal. Lower panel: Whole mounts of longitudinal muscle with adherent myenteric ganglia. No loss of VIP fibers orally to the lesion. Anally there was a loss of fibers for 1-2 mm, followed by a gradual increase in the number of fibers up to 4 mm; beyond this point the fiber frequency was normal. X175 (upper panel), X200 (lower panel).
- Fig. 6. Galanin nerve fibers in rat colon, 10 days after intestinal clamping. Upper panel: Cryostat sections, mucosa and submucosa visible. No obvious change in the number of fibers orally or anally to the lesion. Lower panel: Whole mounts of longitudinal muscle with adherent myenteric ganglia. Orally to the lesion there was no loss of galanin fibers. There was a loss of fibers for 3 mm anally to the lesion. Beyond this point there was a gradual increase in the number of fibers and a normal frequency could be seen at approx. 6 mm anally to the lesion. (A moderately immunoreactive nerve cell body is marked by asterix). X150 (upper panel), X200 (lower panel).

Fig. 7. CGRP nerve fibers in rat colon, 10 days after intestinal clamping (upper panel) or myectomy (lower panel). Upper panel: Cryostat sections, mucosa and submucosa visible. Loss of CGRP fibers for approx. 3 mm in both directions from the lesion. Beyond these points there was a gradual increase in the number of fibers and a normal frequency could be seen at 5-6 mm both orally and anally to the lesion. Lower panel: Whole mounts of longitudinal muscle with adherent myenteric ganglia. No loss of CGRP fibers orally but loss of fibers for 3 mm anally to the lesion. Further anally the number of CGRP fibers increased gradually and was back to normal at approx. 5 mm from the lesion. (A weakly immunofluorescent large cell body with a closely applied CGRP fiber is marked by asterix). X150 (upper panel), X200 (lower panel).

Fig. 8. Concentrations of (A) CGRP, (B) GRP, (C) SP and (D) Met-enkephalin immunoreactive material (expressed as pmol equivalents of the standard peptide per g wet weight) in 5 mm segments of the rat colon taken orally and anally to the site of the intestinal clamping (indicated by arrow). Values are means $\pm$ SE, n=5.

Fig. 9. GRP fibers in rat colon, 10 days after intestinal clamping. Upper panel: Cryostat sections, mucosa and submucosa visible. Loss of GRP fibers for 1-2 mm from the lesion in both directions. Further away the fibers gradually increased in number. The frequency was back to normal at approx. 3 mm from the lesion in both directions. Lower panel: Whole mounts of longitudinal muscle with adherent myenteric ganglia. No loss of GRP fibers orally to the lesion. Anally there was a loss of fibers up to 6 mm. From 6 to 10 mm from the lesion the fibers gradually increased in number and beyond this point the frequency was back to normal. X200.

Fig. 10. NPY/VIP fibers as represented by NPY immunoreactive fibers, in rat colon, 10 days after intestinal clamping. Cryostat sections of the whole wall. No loss of mucosal NPY/VIP fibers. Myenteric NPY/VIP fibers were unaffected orally to the lesion, anally there was a loss of fibers for 1-2 mm. Further distally the fibers gradually increased in frequency and at 4 mm the number was back to normal. X125.

Fig. 11. SP fibers (upper panel) and enkephalin fibers (lower panel) in rat colon, 10 days after myectomy. Whole mounts of longitudinal muscle with adherent myenteric ganglia. Upper panel: No loss of fibers anally to the lesion. Orally there was a loss of fibers for approx. 3 mm. Beyond this point the number of fibers gradually increased and was back to normal at approx. 5 mm orally. Lower panel: No loss of enkephalin fibers anally to the lesion. There was a loss of fibers for 3 mm orally to the lesion. Further orally the number of enkephalin fibers gradually increased and was back to normal at approx. 5 mm. X200.

Table 1. Details of the antisera

Antigen	code	raised against	Cryostat sections	Working dilution	Source	Reference
VIP	7852	unconjugated pure natural porcine VIP	1:640	1:320	Milab, Malmö Sweden	9
NPYa)	8404	unconjugated synthetic porcine NPY	1:320	1:160	Milab, Malmö Sweden	12
Met-enkephalin Met-enk b)		protein-conjugated synthetic met-enkephalin	1:320	1:160	R.J. Miller and K.J. Chang Burroughs Wellcome Research Triangle Park, N.J. USA	1, 10
	8107	protein-conjugated synthetic met-enkephalin	1:160	1:80	Milab, Malmö Sweden	-
Substance P	SP8	protein-conjugated synthetic bovine substance P	1:320	1:160	P.C. Emson, Cambridge, U.K.	2
CGRP	8427	protein-conjugated synthetic CGRP	1:640	1:320	Milab, Malmö, Sweden	31
Galanin	8416	unconjugated synthetic porcine galanin	1:320	1:160	Milab, Malmö Sweden	11
GRP	R-6902	protein-conjugated synthetic porcine GRP	1:640	1:320	N. Yanaihara, Shizuoka, Japan	27

a) This antiserum does not cross-react with avian pancreatic polypeptide, bovine pancreatic polypeptide, peptide YY, VIP or PHI.

b) The two met-enkephalin antisera recognize also leu-enkephalin and produced identical results.

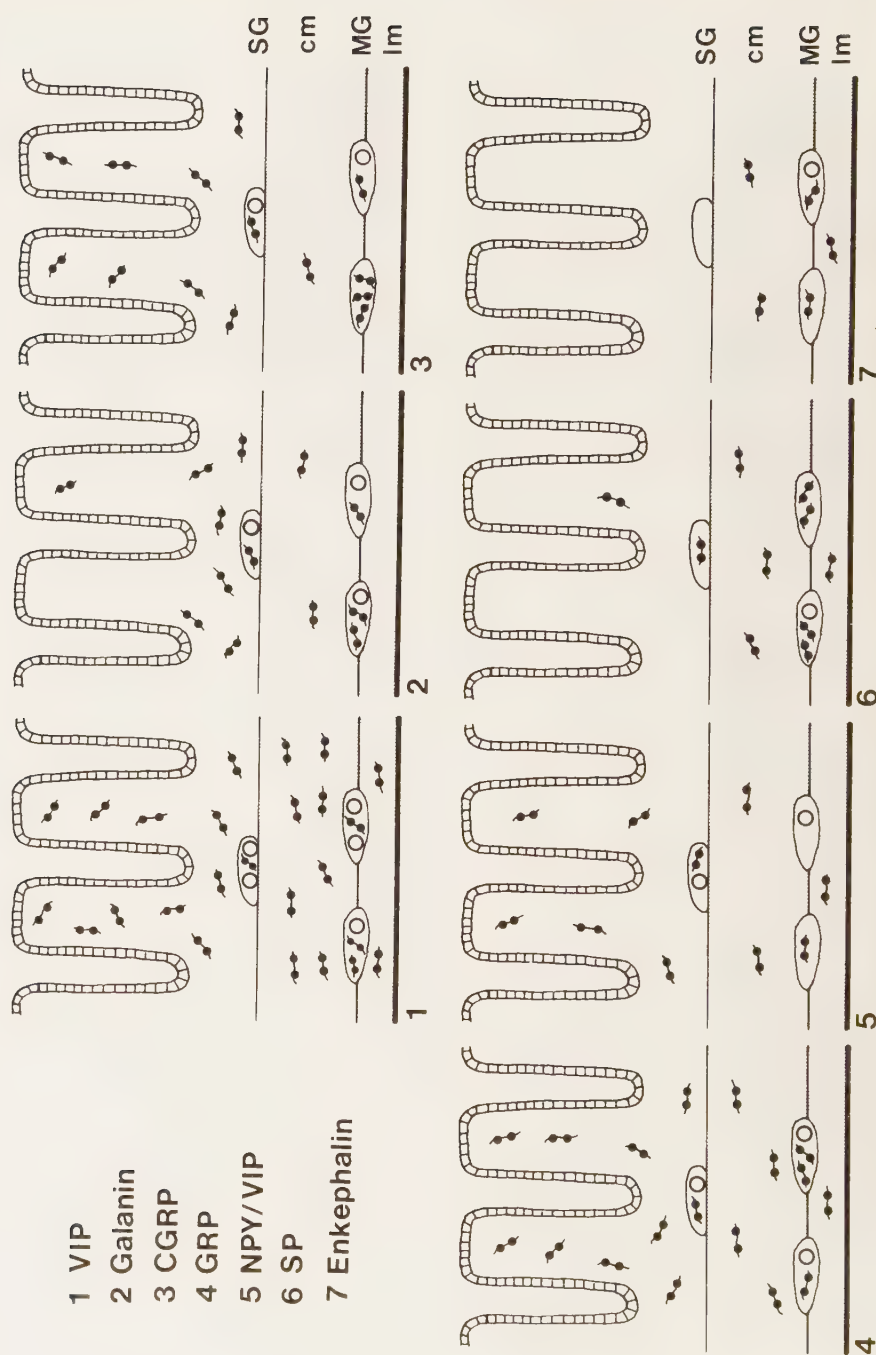


Fig. 1



Fig. 2

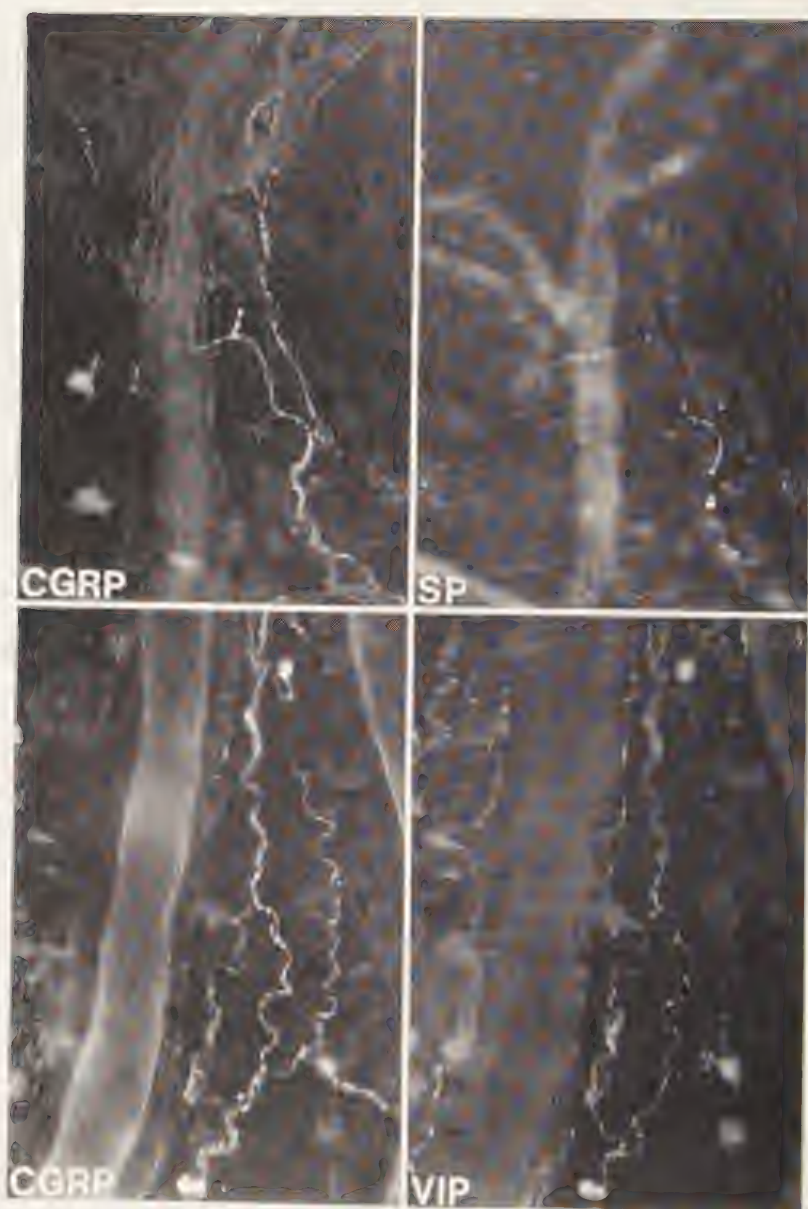


Fig. 1

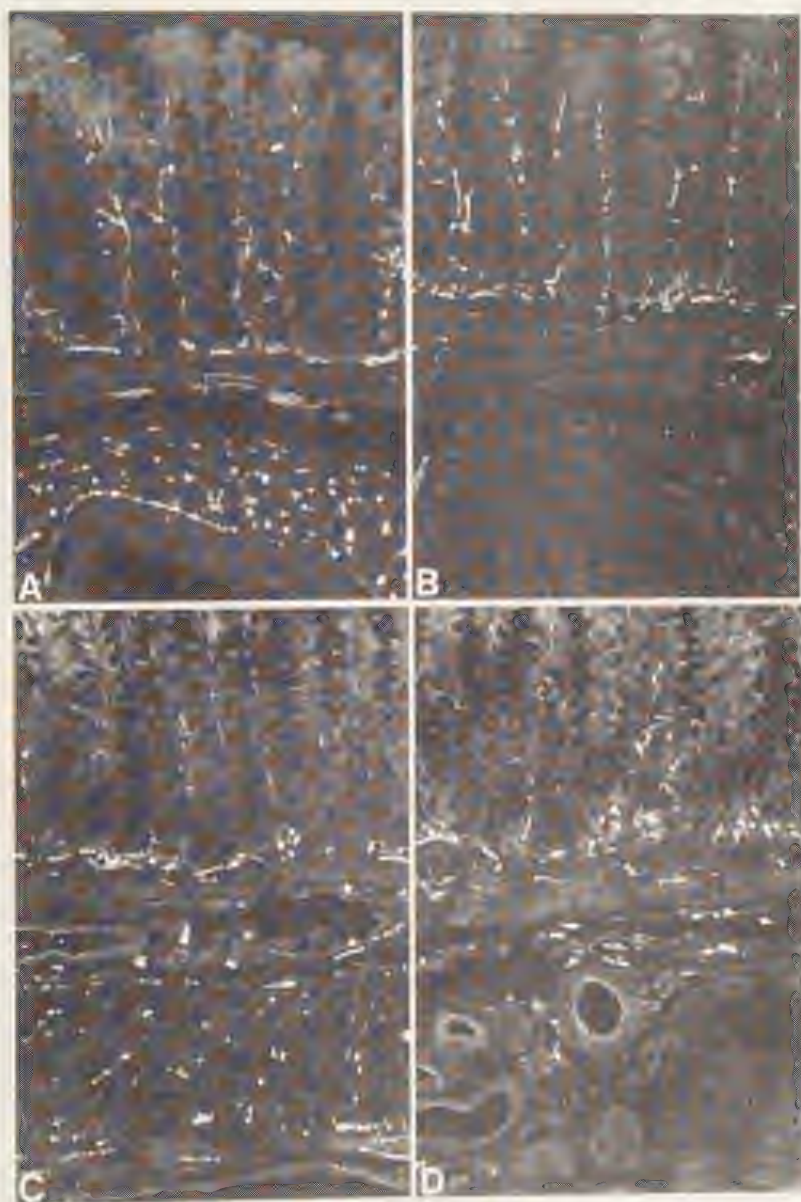


Fig. 4

VIP



3mm



1mm

← Orally Anally →



1mm



1mm

← Orally Anally →



2mm



4mm

Fig-5

Galanin

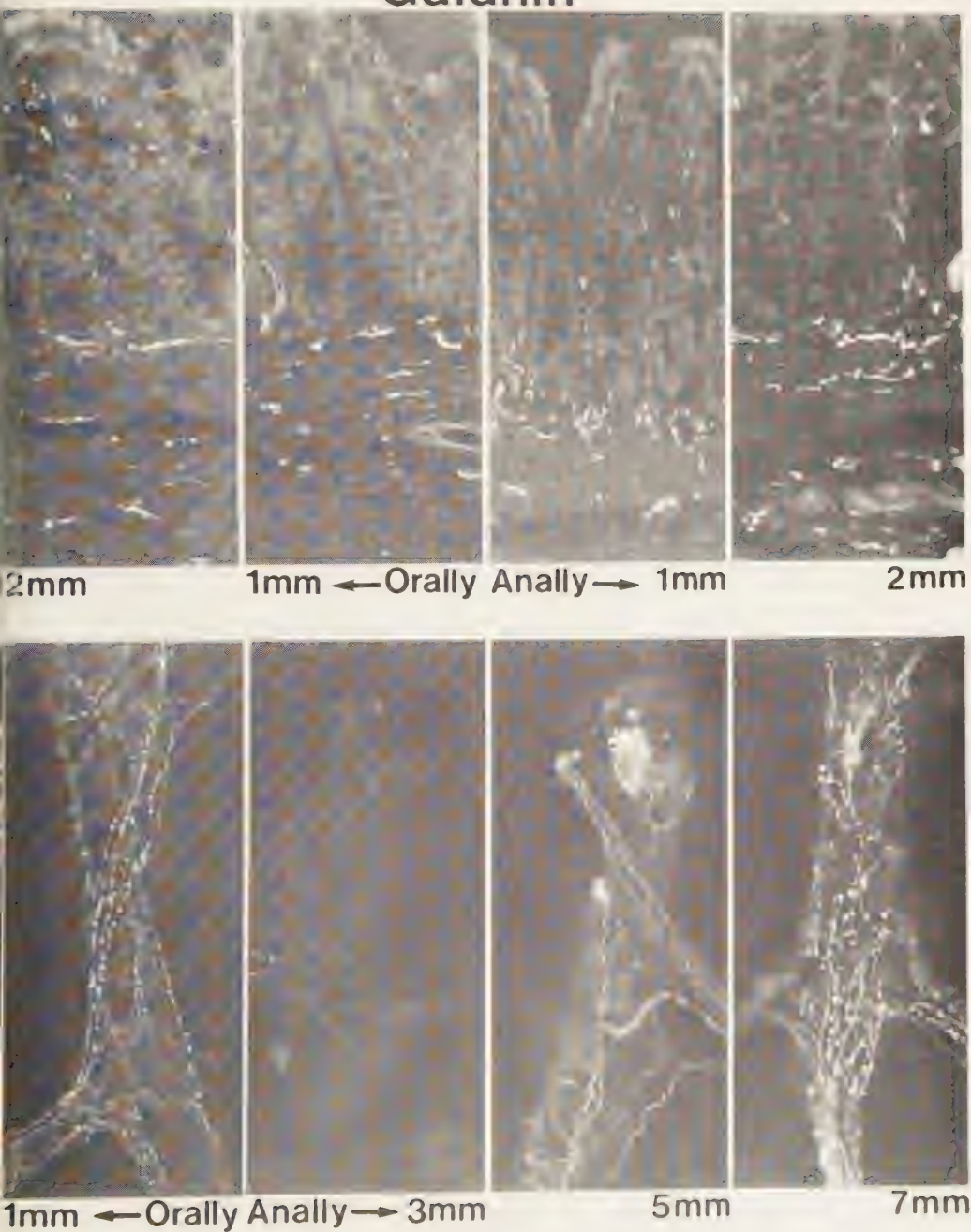


Fig. 6

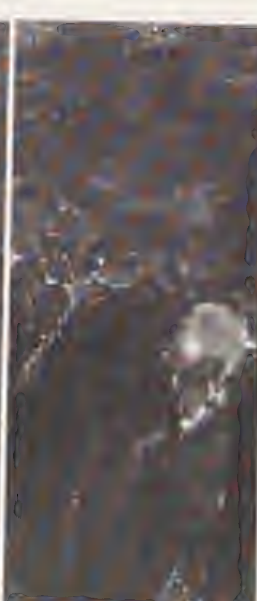
CGRP



6mm

3mm ← Orally Anally → 3mm

6mm



1mm ← Orally Anally → 3mm

4mm

5mm

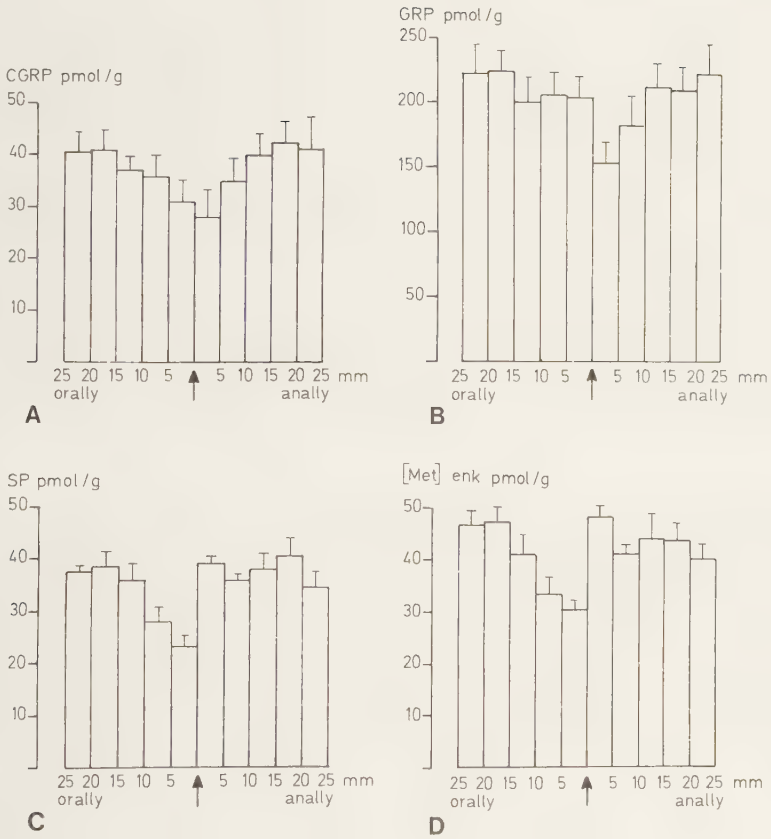


Fig. 8

GRP



4mm



2mm ← Orally Anally → 2mm



4mm



1mm ← Orally Anally → 6mm



8mm



10mm

NPY/VIP

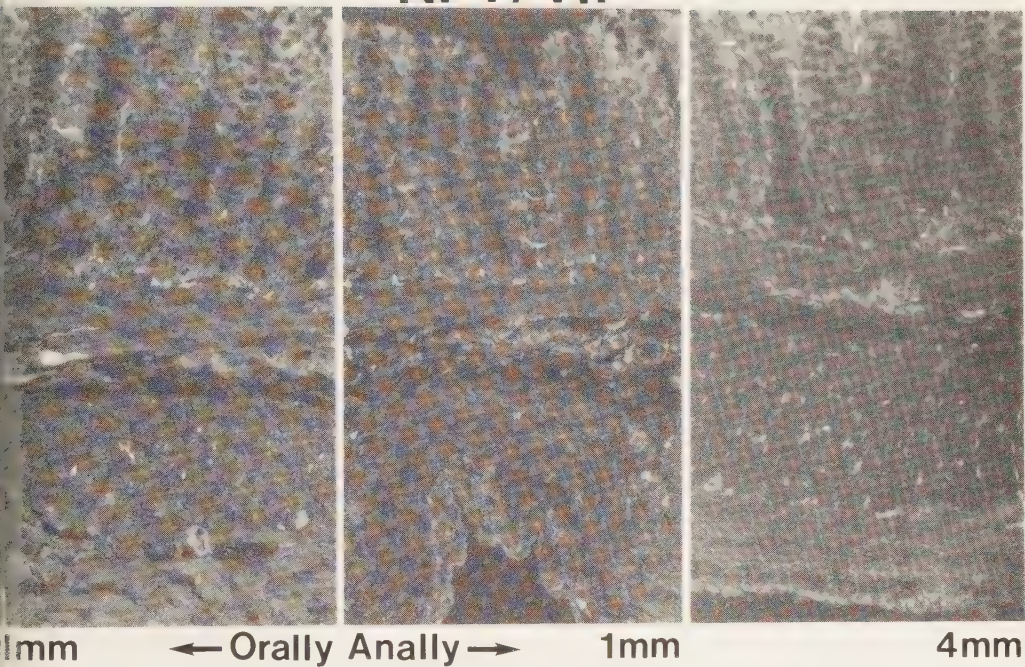


Fig.10

SP



5mm



3mm



1mm

← Orally Anally →

Enkephalin



5mm



3mm



1mm

← Orally Anally →

Reinnervation in the gut: Return of GRP nerve fibers in rat small intestine
after local removal of myenteric ganglia

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Short title: Return of enteric GRP nerves

Key words: gut innervation, enteric neurons, neuropeptides, gastrin-releasing
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Abstract

Gastrin releasing peptide (GRP) is a neuropeptide with a wide distribution in the rat small intestine. Most of the GRP-containing fibers are intramural in origin. Local severing of myenteric GRP neurones by circumferential removal of the outer longitudinal muscle layer together with the adherent myenteric ganglia (myectomy) in a segment of the rat jejunum resulted in the disappearance of GRP fibers from the myectomized circular muscle and from myenteric ganglia and both muscle layers for approximately 10 mm anally to the lesion. As examined at different time intervals up to 60 weeks postoperatively fine-varicose GRP fibers of a normal appearance were found to gradually reinnervate the portion anally to the lesion beginning at 20 weeks, first in the more distal portions and then (after 40-60 weeks) also in the more proximally located portions. Also the circular muscle in the myectomized segment became reinnervated during this time period. Here, however, the fibers were notably coarse, more numerous than in control circular muscle, and arranged in thick bundles (hyperinnervation). Such nerve bundles were most frequent 40 weeks after the operation. The results indicate a remarkable plasticity of enteric neurons.

Introduction

Gastrin releasing peptide (GRP) is a 27 amino acid bombesin-like peptide originally isolated from porcine stomach (11). GRP/bombesin-like immunoreactivity has been demonstrated in neuronal elements in the gut of a number of species (3, 4, 8, 9, 10, 12). The fibers predominate in the smooth muscle and myenteric ganglia although they occur in all layers of the gut wall and the presence of GRP immunoreactive nerve cell bodies in the intramural ganglia indicates that the bulk of GRP fibers are intrinsic in origin (5, 12). In the small intestine of rat and guinea pig myenteric GRP neurones issue descending projections approximately 15-20 mm in length (3, 5). This was established by examining the loss of GRP fibers after axonal degeneration upon local severing of intramural nervous pathways. One such procedure involves removal of approx. 10 mm of the outer longitudinal muscle layer with adherent myenteric ganglia (myectomy). As examined in the rat 1-5 weeks later this resulted in depletion of GRP fibers from the myectomized circular muscle and from the myenteric ganglia and both muscle layers up to 10 mm anally to the lesion. Further distally there was a gradual increase in the number of fibers; normal fiber frequency was observed 20-25 mm anally to the lesion. Orally to the lesion a slight reduction of GRP fibers could be noted in the first 2 mm only, further orally the GRP innervation was intact (5).

Little is known about the plasticity of gut intramural neurones including their capacity for reinnervation. The aim of the present study was to examine the long term effects of myectomy on GRP fibers in lesioned and adjacent segments of the rat small intestine.

Materials and Methods

60 female rats (150-170 g) were subjected to small intestinal myectomy i.e. circumferential removal of 10 mm of the outer longitudinal smooth muscle layer with adherent myenteric ganglia at the mid-jejunal level (7). The animals were

left for 8-10 days, 5, 10, 20, 40 or 60 weeks after the operation (10 in each group). They were killed by decapitation and specimens were taken from the myectomized segment together with specimens orally and anally to the lesion. The tissue specimens were processed either for immunocytochemistry or radioimmunoassay of GRP. In addition, 5 unoperated rats were used to study the normal distribution of GRP fibers at the mid-jejunal level.

Immunocytochemistry

Tissue specimens from the jejunum of unoperated and operated rats were opened along the mesenteric border, orientated in such a way that the oral and anal ends could be identified, and pinned flat on a piece of balsa wood with the mucosal side facing the wood. The preparations were fixed by floating in a mixture of 2% formaldehyde and 15% of a saturated aqueous picric acid solution in 0.1 M phosphate buffer (pH 7.2) overnight and thoroughly rinsed in Tyrode buffer containing 10% sucrose. The specimens were processed for immunocytochemistry either as whole mounts or after cryostat sectioning. The outer (longitudinal), inner (circular) muscle layers and the submucosa were separated and placed on chrome-alum-coated slides as whole mounts (2). Before further processing the whole mounts were dried, frozen and thawed in order to facilitate antibody penetration. Alternatively, specimens were frozen on dry ice and longitudinal sections were cut in a cryostat at 10 μ m thickness. For the immunocytochemical demonstration of GRP we used a previously characterized rabbit antiserum (code no. R-6902, kindly donated by Prof. N. Yanaihara, Shizuoka, Japan) in dilution 1:640 (10, 15). The site of the antigen-antibody reaction was revealed by the indirect immunofluorescence method (1). For controls we used antiserum which had been inactivated by the addition of excess amounts of antigen (10-100 μ g synthetic GRP per ml diluted antiserum) (Peninsula, Belmont, CA., USA). The antiserum was inactivated by bombesin (Peninsula, Belmont, CA., USA) but did not cross-react with other neuropeptides, such as substance P,

neurokinin A, physalaemin, Leu- and Met-enkephalin, VIP, neurotensin (Peninsula, Belmont, CA., USA) or somatostatin (Ferring, Malmö, Sweden) (10-100 µg synthetic peptide per ml diluted antiserum). However, cross-reaction with other peptides or proteins containing amino acid sequences recognized by the antiserum cannot be excluded. It is appropriate, therefore, to refer to the immunoreactive material as GRP-like rather than GRP. For simplicity, the shorter term is used in the following.

Radioimmunoassay

The concentrations of GRP in extracts of intestinal specimens were determined by radioimmunoassay. The jejunal segments were opened, rinsed and cut serially in 5 mm specimens, 4 orally and 5-6 anally to the lesion. The specimens were weighed and boiled in 0.5 M acetic acid (0.1 g wet weight/ml) for 15 min. The tissue was homogenized (Polytron 1-2 min). After centrifugation at 2000 xg for 15 min the supernatants were collected and lyophilized. The freeze-dried material was taken up in 2 ml of 0.05 M phosphate buffer (pH 7.4) containing 0.25% human serum albumin. Details of the radioimmunoassay of GRP have been given elsewhere (12). Briefly, bombesin antiserum (code no. 8050, Milab, Malmö, Sweden) was used in dilution 1:14000. Under the assay condition this antiserum cross-reacts with GRP (30%) and GRP 14-27 (37%) indicating that the antibodies recognize the C-terminal portion of bombesin which is shared by GRP. Bombesin, GRP and GRP 14-27 were purchased from Peninsula, Belmont, CA. The concentrations were expressed as pg GRP equivalents per g wet weight and recalculated as percent of the concentration in the most proximal segment.

Results

Unoperated rats

Fine-varicose intensely immunoreactive GRP fibers were numerous in the myenteric ganglia (Fig. 1A). They were moderate in number in the circular and longitudinal muscle layers (Fig. 1B). Scattered, moderately immunoreactive GRP fibers occurred in the mucosa and submucosa, predominantly around the basal part of the crypts (Fig. 1C). GRP immunoreactive nerve cell bodies were regularly seen in the myenteric ganglia but only rarely in the submucous ganglia (Fig. 1A and D). Radioimmunoassay revealed a concentration of 92.3 ± 9.7 ng GRP equivalents/g wet weight in whole wall specimens (mean $\pm$ SEM, $n=35$).

Myectomized rats

The time-course of the effects of myectomy on GRP innervation orally and anally to the lesion is illustrated in Figs. 2 and 3. The simultaneous effects in the myectomized segment are illustrated in Figs. 4 and 5. An attempt to illustrate semiquantitatively the time course of the effects of myectomy on GRP fiber frequency is given in Fig. 6.

8-10 days, 5 and 10 weeks. In these three groups GRP nerve fibers were absent from the myenteric ganglia and smooth muscle anally to the lesion for a distance of approximately 10 mm. Further distally, a gradual rise in the number of GRP fibers could be observed up to 25 mm; beyond this point the fibers had a normal frequency (Fig. 2 and 6). 8-10 days and 5 weeks but not 10 weeks post-operatively there was a slight reduction in the number of GRP fibers in the myenteric ganglia less than 2 mm orally to the lesion. Further orally, the frequency of GRP fibers was normal. At all these stages GRP fibers were lacking in the circular muscle of the myectomized segment (Fig. 4A). In the mucosa and submucosa the GRP innervation was intact both orally and anally to the lesion as well as in the myectomized segment. Radioimmunoassay revealed a notably low

GRP concentration in the myectomized segment and markedly reduced concentrations up to 20-25 mm anally. There was no reduction in the segments taken orally to the lesion (Fig. 7A, B).

20 weeks. In the myenteric ganglia and smooth muscle GRP nerve fibers were lacking anally to the lesion for a distance of approximately 5 mm. Further distally the number of immunoreactive fibers increased gradually; the GRP fiber frequency was back to normal at a distance of approximately 15 mm from the lesion (Fig. 2 and 6). Orally to the lesion the frequency of GRP fibers was normal. Scattered bundles of coarse GRP fibers could be detected in the myectomized circular muscle (Fig. 4B and 5A), while the GRP innervation in the mucosa and submucosa was intact. Radioimmunoassay revealed a reduced GRP concentration in the segments anally to the lesion, however not as marked as after 8-10 days or 5 weeks. In the myectomized segment the GRP concentration was higher than shortly after the operation (Fig. 7C).

40 weeks. GRP fibers were absent from the myenteric ganglia and smooth muscle for a distance of 2-3 mm anally to the lesion. Further distally, the frequency of GRP fibers increased gradually and was back to normal at approximately 10 mm from the lesion. Orally to the lesion the fiber frequency was normal (Fig. 3 and 6). Numerous coarse GRP immunoreactive fibers were seen to run in bundles in the myectomized circular smooth muscle (Fig. 4C and D, and 5B). The nerve fiber bundles formed a meshwork; the orientation of the bundles had no obvious relation to the organization of the smooth muscle cells. There was no overt change in the frequency of mucosal and submucosal GRP fibers. However, scattered coarse GRP fibers could be observed together with delicate, beaded GRP fibers of normal appearance (Fig. 5D). Radioimmunoassay revealed a markedly

higher GRP concentration in the myectomized segment than at earlier times studied. Anally to the myectomy the concentration of GRP was reduced only in the first 5 mm segment (Fig. 7D).

60 weeks. GRP fibers were absent from the myenteric ganglia and smooth muscle for a distance of 2-3 mm anally to the lesion. The fibers gradually increased in number further anally; sometimes markedly thickened fibers were seen. 6-8 mm anally the frequency and appearance of the GRP fibers was normal. Orally to the lesion the GRP innervation was normal (Fig. 3 and 6). Bundles of coarse GRP fibers were common in the myectomized circular smooth muscle (Fig. 4E and F, and 5C). These bundles intermingled with single varicose GRP fibers of normal appearance. The GRP fibers in the mucosa and submucosa had a normal frequency and appearance. As after 40 weeks radioimmunoassay revealed a high GRP concentration in the myectomized segment; the concentration of GRP remained low in the first 5 mm segment anally to the lesion (Fig. 7E).

Discussion

Previous studies in several mammals have shown GRP nerve fibers to occur in all layers of the gut with a predominance in the myenteric ganglia and smooth muscle (4, 8, 9, 12). Myenteric GRP neurones in the rat and guinea pig small intestine issue descending projections approximately 20 mm in length (3, 5). These projections are "long" compared with most other neuronal populations, such as those storing substance P, vasoactive intestinal polypeptide/neuropeptide Y, somatostatin or calcitonin gene related peptide which issue projections shorter than 8 mm (6, 7). Mucosal GRP fibers originate in the submucous ganglia and project in both oral and anal direction for approximately 2 mm in the rat (as revealed by intestinal clamping) (6, Ekblad et al, unpublished). In the present study we examined both short and long term effects of myectomy on the GRP innervation of rat small intestine. Shortly after the myectomy fibers

from myenteric GRP neurons had disappeared anally to the lesion for a distance of approximately 10 mm in agreement with previous findings (5). After 20 weeks GRP fibers began to gradually reappear anally to the lesion, first in the more distal portion and then (after 40-60 weeks) also in the more proximally located portions. However, immediately adjacent to the lesion a normal GRP fiber frequency was not established, not even after 60 weeks. A few of the returning GRP fibers were notably coarse but the vast majority distally to the lesion had a normal fine-varicose appearance. In the myectomized circular muscle there was an initial loss of GRP fibers (c.f. 5). After 20 weeks GRP fibers had returned in this area but they were all notably coarse, running in thick bundles. Such bundles were most common 40 weeks after the operation. 60 weeks after the myectomy they persisted, although somewhat reduced in number; at this time normal-looking fine-varicose GRP fibers could be seen alongside the more coarse ones. GRP fibers remained at all times in the mucosa and submucosa of the myectomized segment. This finding differs slightly from that in a previous report (5) and may reflect a somewhat better preservation of the immunoreactivity of GRP in nerve fibers by the fixative used in the present study allowing detection also of the most delicate mucosal nerve fibers.

On the whole the immunocytochemical findings agreed well with the results of the chemical GRP determinations in that there was a markedly reduced GRP concentration anally to the lesion up to 20 weeks postoperatively followed by gradually increasing concentrations 40 and 60 weeks postoperatively. Further, there was a notably low GRP concentration in the myectomized segment of the jejunum 8-10 days and 5 weeks postoperatively followed by markedly increased GRP concentrations 20, 40 and 60 weeks postoperatively. Thus, GRP fibers returned both in the myectomized circular muscle and in the myenteric ganglia and muscle layers anally to the lesion. Interestingly, the pattern of reinnervation in these two locations seemed to be different; in the myectomized circular muscle the GRP nerve fibers were coarse, arranged in bundles and more numerous

than normally, suggesting hyperinnervation. In the muscle layers and myenteric ganglia anally to the lesion the gradually reappearing GRP fibers had a normal morphology, and hyperinnervation was not noted. It may be speculated that the discrepancy reflects the different situations in the two regions displaying reinnervation. Thus, the myectomized circular muscle is denervated in the sense that it is cut off from myenteric ganglia (and consequently from GRP cell bodies). The region anally to the lesion probably harbours the normal number of myenteric neurones (including GRP cell bodies) but lacks nerve fibers (including GRP fibers) projecting down stream from the point of lesion. Interestingly, local removal of myenteric nerve cell bodies (as in the myectomized segment) resulted in hyperinnervation with bundles of notably coarse fibers, whereas local elimination of descending nerve fibers was followed by a gradual return of normal GRP fibers, beginning in the most distal segment. Some mucosal fibers in the myectomized segment were notably coarse 40 weeks after the operation despite the fact that the mucosal GRP nerve supply was not directly affected by the operation. Possible explanations for the reinnervation and hyperinnervation phenomena are illustrated in Fig. 8. The coarse nerve fibers and their arrangement in thick bundles in the hyperinnervated circular muscle are reminiscent of the situation in the afflicted segments in Crohn's disease and Hirschsprung's disease (aganglionosis) (10, 13, 14).

In conclusion, the results of the present study indicate a remarkable plasticity of intramural GRP neurones in the rat small intestine. The functional significance of the phenomenon of reinnervation and hyperinnervation in the gut wall is yet to be explained.

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References

1. Coons A.H., Leduc E.H. and Connolly J.M. (1955) Studies on antibody production. I. A method for the application to a study of the hyperimmune rabbit. *J. Exp. Med.* 102, 49-60.
2. Costa M., Buffa R., Furness J.B. and Solcia E. (1980) Immunohistochemical localization of polypeptides in peripheral autonomic nerves using whole mount preparations. *Histochemistry* 65, 157-165.
3. Costa M., Furness J.B., Yanaihara N., Yanaihara C. and Moody T.W. (1984) Distribution and projections of neurons with immunoreactivity for both gastrin-releasing peptide and bombesin in the guinea-pig small intestine. *Cell Tissue Res.* 235, 285-293.
4. Dockray G.J., Vaillant C. and Walsh J.H. (1979) The neuronal origin of bombesin-like immunoreactivity in the rat gastrointestinal tract. *Science, N.Y.* 4, 1561-1568.
5. Ekblad E., Ekman R., Håkanson R. and Sundler F. (1984) GRP neurones in the rat small intestine issue long anal projections. *Regul. Pept.* 9, 279-287.
6. Ekblad E., Winther C., Ekman R., Håkanson R. and Sundler F. (1986) Projections of peptide-containing neurones in rat small intestine. *Neuroscience* in press.
7. Furness J.B., Costa M., Llewellyn-Smith I.J., Franco R. and Wilson A.J. (1981) Polarity and projections of peptide-containing neurons in the guinea pig small intestine. In: *Cellular Basis of Chemical Messengers in the Digestive System*, (eds. Grossman M.I., Brazier M.A.B. and Lechago J.) Academic Press, New York, no 23 pp. 201-213.
8. Iwanaga T. (1983) Gastrin-releasing peptide (GRP)/bombesin-like immunoreactivity in the neurons and paraneurons of the gut and lung. *Biomed. Res.* 4, 93-104.

9. Leander S., Ekman R., Uddman R., Sundler F. and Håkanson R. (1984) Neuronal cholecystokinin, gastrin-releasing peptide, neurotensin and β -endorphin in guinea pig intestine. Distribution and possible motor functions. *Cell Tissue Res.* 235, 521-531.
10. Larsson L.T., Malmfors G. and Sundler F. (1983) Peptidergic innervation in Hirschsprung's disease. *Arch. Kinderchir.* 38, 301-304.
11. McDonald T.J., Jörnvall H., Nilsson G., Vagne M., Ghatei M., Bloom S.R. and Mutt V. (1979) Characterization of a gastrin releasing peptide from porcine non-antral gastric tissue. *Biochem. Biophys. Res. Commun.* 90, 22-233.
12. Moghimzadeh E., Ekman R., Håkanson R., Yanaihara N. and Sundler F. (1983) Neuronal gastrin-releasing peptide in the mammalian gut and pancreas. *Neuroscience* 10, 553-563.
13. Polak J.M. and Bloom S.R. (1978) Peptidergic nerves of the gastrointestinal tract. *Invest. Cell Pathol.* 1, 301-326.
14. Sjölund K., Schaffalitzky de Muckadell O.B., Fahrenkrug J., Håkanson R., Peterson B.G. and Sundler F. (1983) Peptide-containing nerve fibres in the gut wall in Crohn's disease. *Gut* 24, 724-733.
15. Yanaihara N., Yanaihara C., Mochizuki T., Imura K., Fujita T. and Iwanaga T. (1981) Immunoreactive GRP. *Peptides* 2 (suppl 2), 185-192.

Legends to figures

Fig 1 Whole mounts (A, B and D) and cryostat section (C) from rat jejunum. (A) Numerous GRP fibers and one nerve cell body (arrow) in the myenteric ganglia. (B) Delicate GRP fibers running in the circular smooth muscle. (C) Delicate GRP fibers in the mucosa ramifying among the crypts. (D) GRP nerve cell body with weakly immunoreactive process in submucous ganglion. A X200, B X250, C X175, D X250.

Fig 2 Whole mounts of longitudinal muscle with adherent myenteric ganglia from both sides of myectomy. Upper panel shows GRP nerve fibers 5 weeks after myectomy. Up to 2 mm orally to the lesion the number of GRP fibers is slightly reduced. Further orally the nerve fiber frequency is normal. GRP fibers are lacking for approx. 10 mm anally to the lesion. Beyond this point there is a gradual return of nerve fibers and at 20-25 mm anally to the lesion the innervation is back to normal. Lower panel shows GRP nerve fibers 20 weeks after myectomy. No loss of fibers orally to the lesion. GRP fibers are absent for a distance of approx. 5 mm anally to the lesion. Further distally the fibers increase gradually in number. Approx. 15 mm from the lesion the number of GRP fibers is back to normal. X175.

Fig 3 Whole mounts of longitudinal muscle with adherent myenteric ganglia from both sides of myectomy. Upper panel shows GRP nerve fibers 40 weeks postoperatively. No loss of fibers orally to the lesion. Anally to the lesion GRP fibers are lacking for approx. 3 mm. Beyond this point GRP fibers gradually return, at approx. 10 mm the fiber frequency is normal. Lower panel shows GRP nerve fibers 60 weeks after myectomy. No loss of fibers orally to the lesion. Anally to the lesion there is a loss of GRP

fibers for approx. 2 mm. Further anally the fibers, sometimes markedly thickened (arrow), gradually increase in number. 6-8 mm anally to the lesion the innervation is back to normal. X175.

Fig 4 Whole mounts of myectomized circular smooth muscle. 5 (A), 20 (B), 40 (C and D) and 60 (E and F) weeks postoperatively. GRP fibers are lacking at 5 weeks. At 20 weeks bundles of weakly immunoreactive GRP fibers are seen. At 40 weeks numerous GRP fibers are seen to run in thick bundles. At 60 weeks the fiber bundles are somewhat less numerous than at 40 weeks. In addition, single varicose GRP fibers (thin or coarse) can be seen. A, B, C and E X180, D and F X225.

Fig 5 Cryostat sections from the myectomized segment showing circular muscle 20 (A), 40 (B) and 60 (C) weeks postoperatively and mucosa 40 (D) weeks postoperatively. Scattered bundles of GRP fibers are seen in the circular muscle at 20 weeks. At 40 weeks these bundles are numerous and notably thick. At 60 weeks GRP nerve fiber bundles are slightly reduced in number and single fine varicose fibers can be seen as well. At 40 weeks both coarse and fine varicose GRP fibers can be seen in the mucosa. A-C X160, D X200.

Fig 6 Schematic illustration of the effects of myectomy on the myenteric GRP innervation orally and anally to the lesion and in the myectomized circular muscle. Four different times after operation (5, 20, 40 and 60 weeks) are represented. The abscissa shows the site of the myectomy (indicated by a horizontal black bar) together with the distances from the lesion (in mm). The ordinate gives the fiber frequency estimated semiquantitatively: + rare fibers, ++ few fibers, +++ moderate number of fibers (normal number), ++++ numerous fibers, +++++ very numerous fibers.

Fig 7 GRP concentration in 5 mm segments of the rat jejunum taken orally and anally to the myectomy. The area of myectomy is indicated by horizontal line. Time after the operation: 8-10 days (A), 5 weeks (B), 20 weeks (C), 40 weeks (D) and 60 weeks (E). The GRP concentration is expressed as percent of the concentration of the most proximally located segment.

Fig 8 Schematic illustration of the proposed descending projections of myenteric GRP neurons and the short term and long term effects of myectomy. A. Normal distribution and polarity of myenteric GRP neurons. B. Local disruption of GRP neuronal pathways by myectomy causes a loss of GRP fibers in the myectomized circular muscle and up to 10 mm anally to the lesion. C. 20-60 weeks after the operation there is a gradual return of GRP nerve fibers both in the area anally to the lesion (delicate fibers with fine-varicose appearance) and in the myectomized circular muscle (coarse fibers running in bundles). Conceivably, the gradual reappearance of fine-varicose fibers anally to the lesion, beginning in the most distal portion of the affected segment, reflects outgrowth of nerve terminals (sprouting?) from the preterminal part of neurons located immediately down stream of the lesion. Hypothetical sources of the coarse GRP fibers appearing in the myectomized circular muscle are suggested to be orally located myenteric nerve cell bodies and/or submucous or extrinsic nerve cell bodies (arrow).

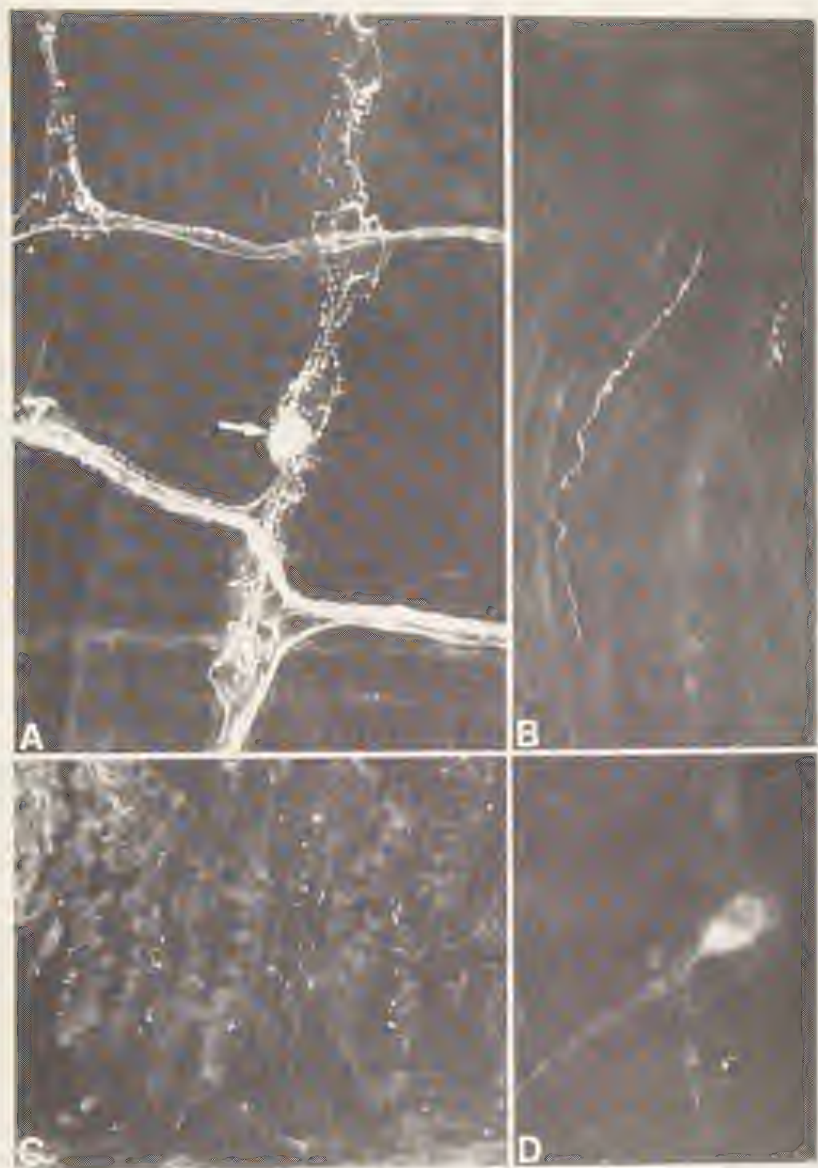


FIG. 1

5 weeks



2mm



15mm



25mm

← Orally Anally →

20 weeks



2mm



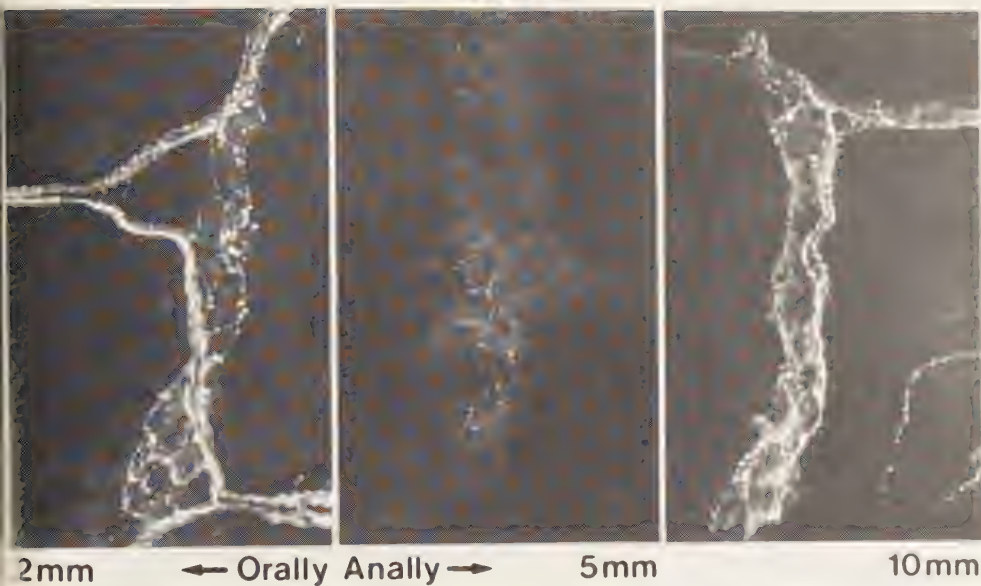
10mm



15mm

← Orally Anally →

40 weeks



60 weeks

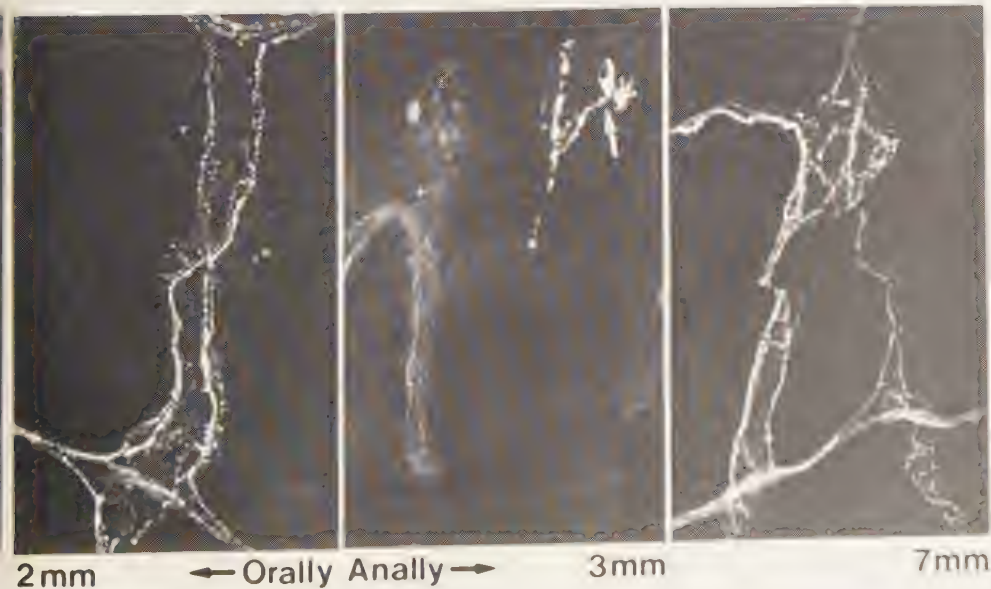


Fig.3

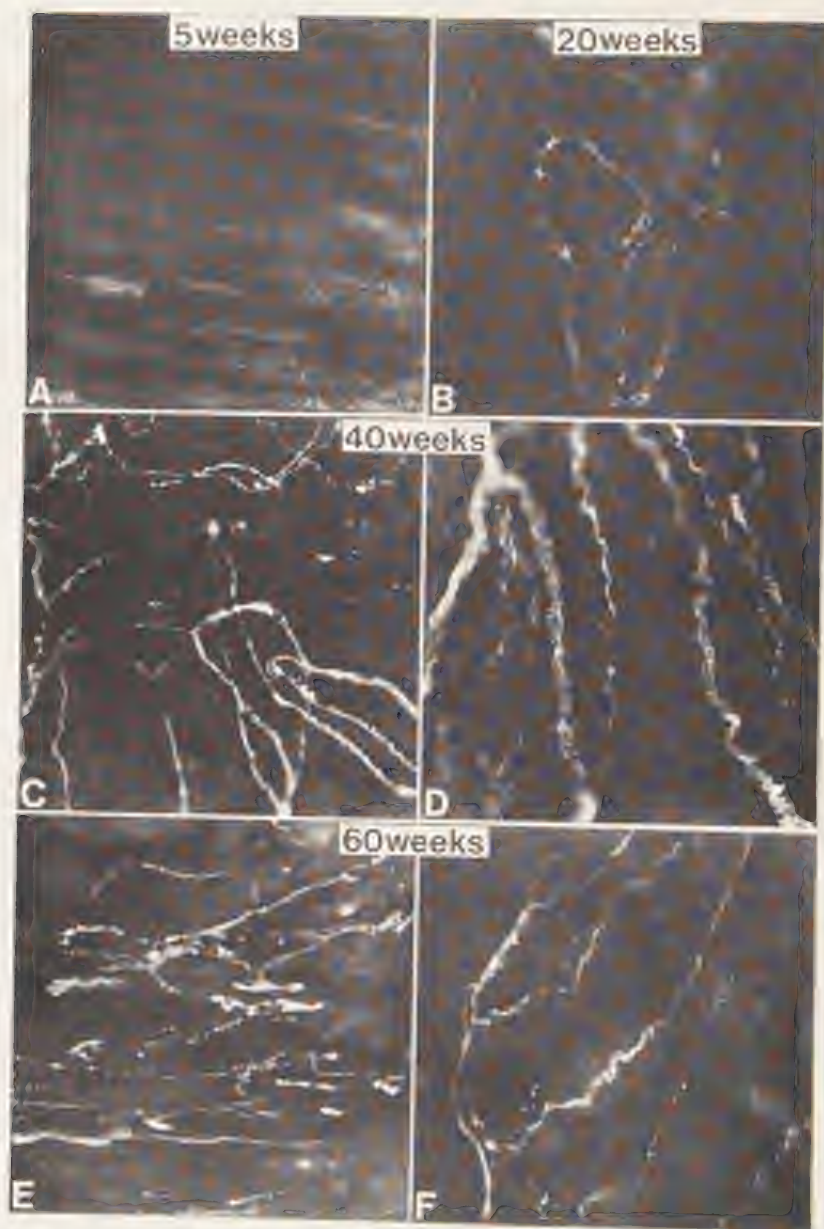


Fig. 4

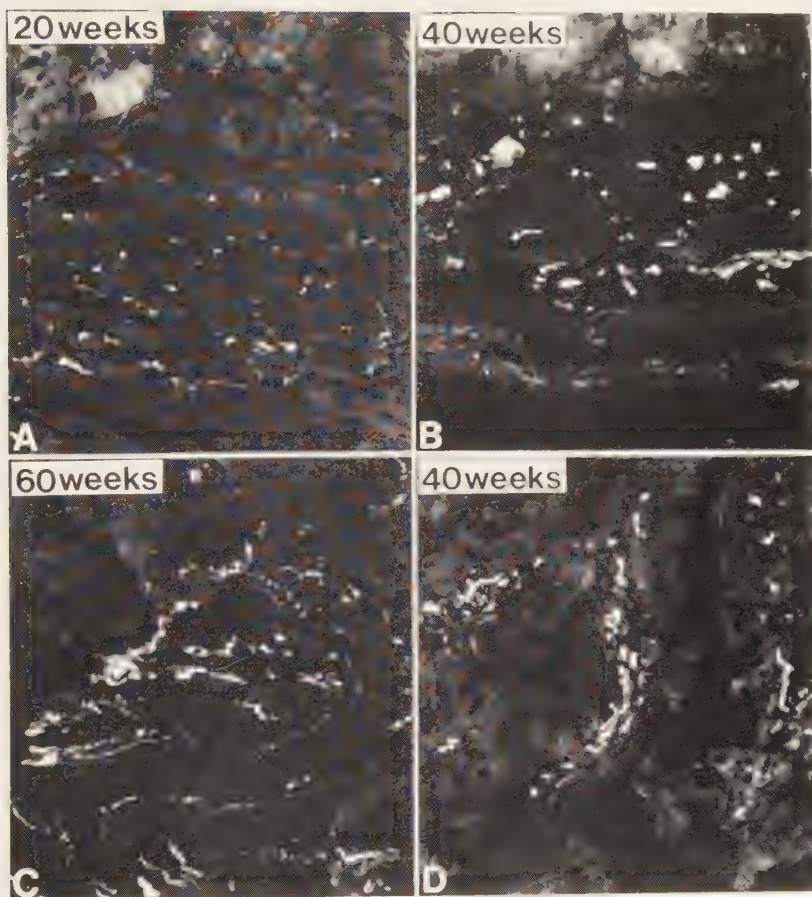


Fig.5

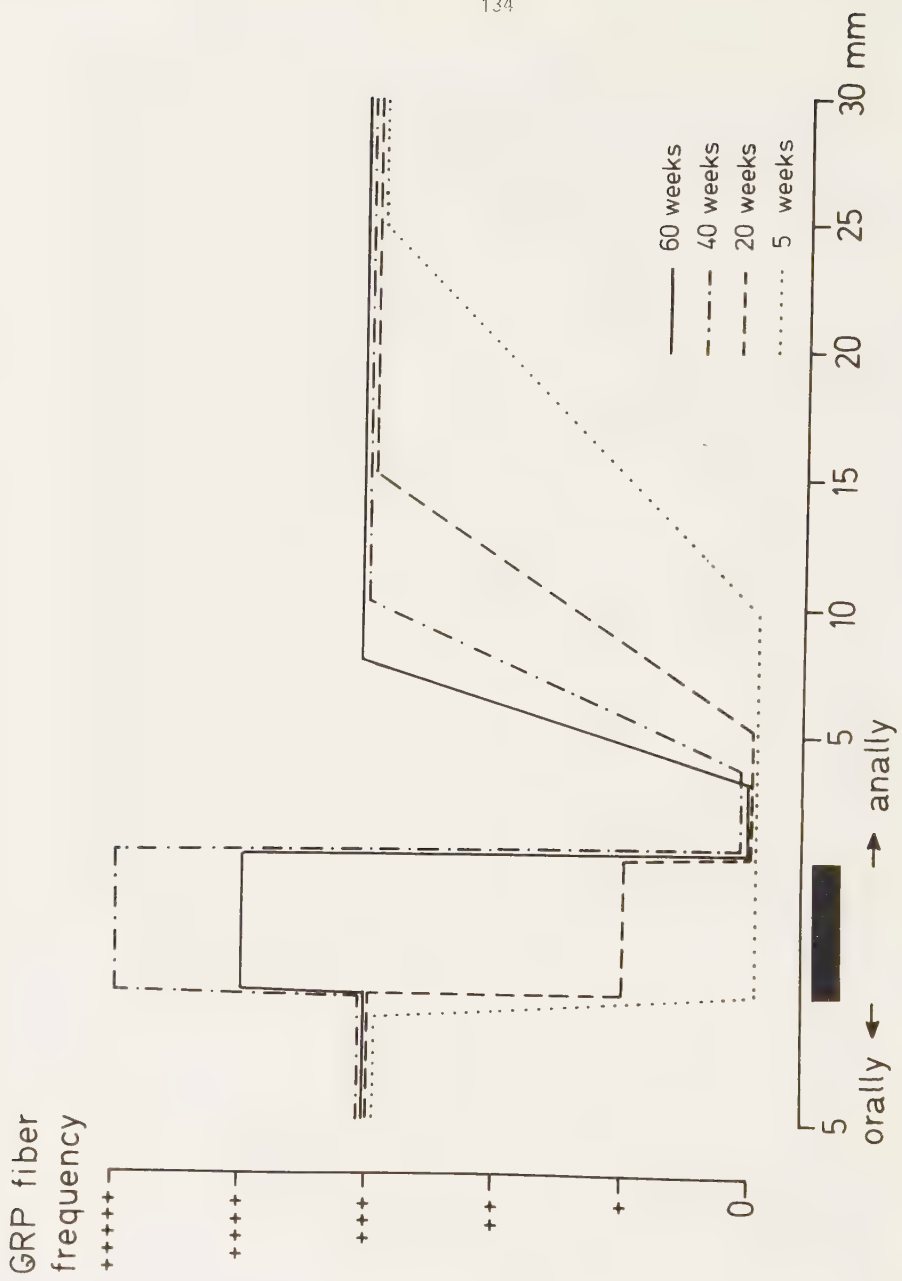


Fig.6

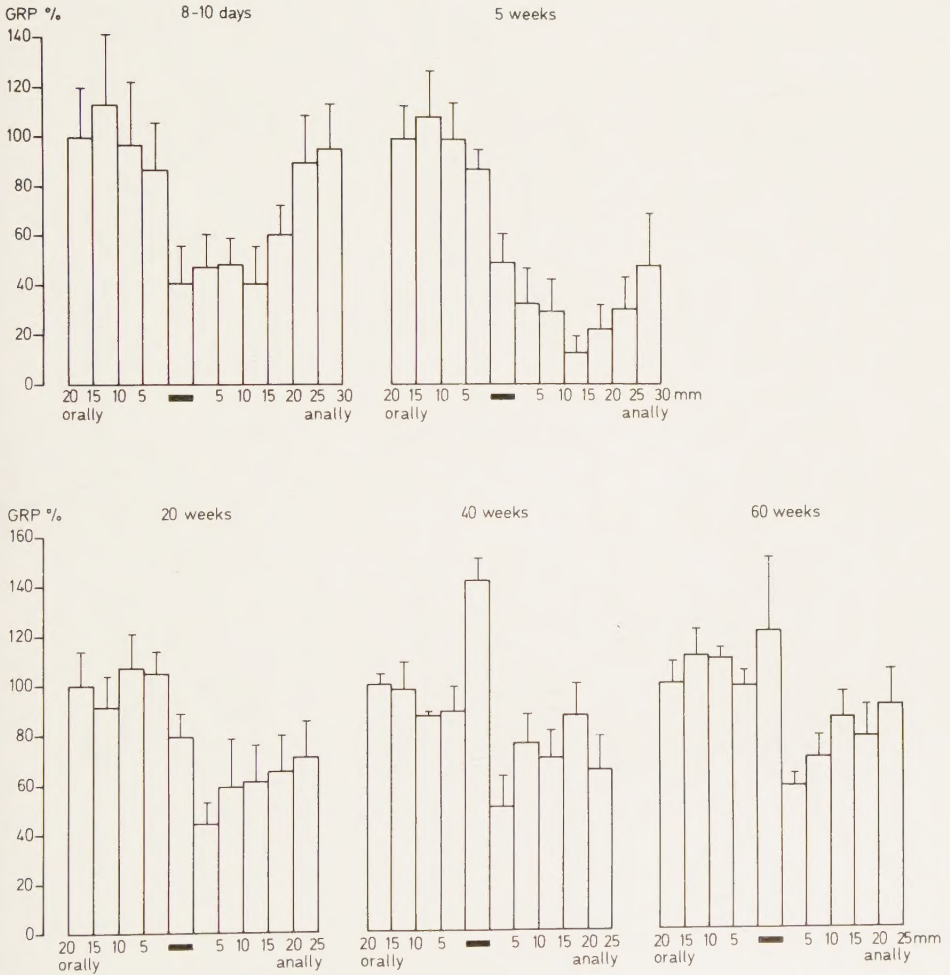


Fig.7

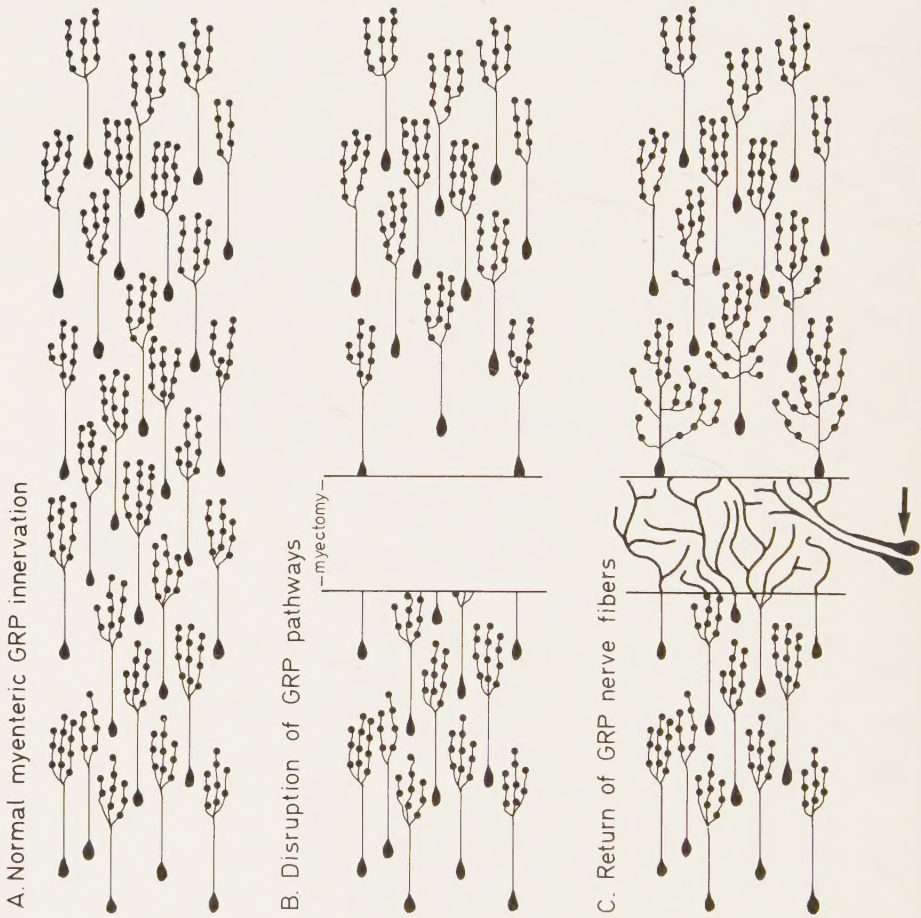


Fig.8

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